Exhibit N

Interaction Trap/Two-Hybrid System to Identify Interacting Proteins

To understand the function of a particular protein, it is often useful to identify other proteins with which it associates. This can be done by a selection or screen in which novel proteins that specifically interact with a target protein of interest are isolated from a library. One particularly useful approach to detect novel interacting proteins—the two-hybrid system or interaction trap (see Figs. 20.1.1 and 20.1.2)—uses yeast as a "test tube" and transcriptional activation of a reporter system to identify associating proteins (see Background Information). This approach can also be used specifically to test complex formation between two proteins for which there is a prior reason to expect an interaction.

In the basic version of this method (see Fig. 20.1.2), the plasmid pEG202 or a related vector (see Fig. 20.1.3 and Table 20.1.1) is used to express the probe or "bait" protein as a fusion to the heterologous DNA-binding protein LexA. Many proteins, including transcription factors, kinases, and phosphatases, have been successfully used as bait proteins. The major requirements for the bait protein are that it should not be actively excluded from the yeast nucleus, and it should not possess an intrinsic ability to strongly activate transcription. The plasmid expressing the LexA-fused bait protein (see Table 20.1.1) is used to transform yeast possessing a dual reporter system responsive to transcriptional activation through the *LexA* operator. In one such example, the yeast strain EGY48 (see Table 20.1.2) contains the reporter plasmid pSH18-34. In this case, binding sites for LexA are located upstream of two reporter genes. In the EGY48 strain, the upstream activating sequences of the chromosomal *LEU2* gene—required in the biosynthetic pathway for leucine (Leu)—are replaced with *LexA* operators (DNA binding sites). pSH18-34 contains a *LexA* operator–*lacZ* fusion gene. These two reporters allow selection for transcriptional activation by permitting selection for viability when cells are plated on medium lacking Leu, and discrimination based on color when the yeast is grown on medium containing Xgal (*UNIT 13.6*).

In Basic Protocol 1, EGY48/pSH18-34 transformed with a bait is characterized for its ability to express protein (Support Protocol 1), growth on medium lacking Leu, and for the level of transcriptional activation of *lacZ* (see Fig. 20.1.2A). A number of alternative strains, plasmids, and strategies are presented which can be employed if a bait proves to have an unacceptably high level of background transcriptional activation.

In an interactor hunt (Basic Protocol 2), the strain EGY48/pSH18-34 containing the bait expression plasmid is transformed (along with carrier DNA made as described in Support Protocol 2) with a conditionally expressed library made in the vector pJG4-5 (see Fig. 20.1.6 and Table 20.1.3). This library uses the inducible yeast *GAL1* promoter to express proteins as fusions to an acidic domain ("acid blob") that functions as a portable transcriptional activation motif (act) and to other useful moieties. Expression of library-encoded proteins is induced by plating transformants on medium containing galactose (Gal), so yeast cells containing library proteins that do not interact specifically with the bait protein will fail to grow in the absence of Leu (see Fig. 20.1.2B). Yeast cells containing library proteins that interact with the bait protein will form colonies within 2 to 5 days, and the colonies will turn blue when the cells are streaked on medium containing Xgal (see Fig. 20.1.2C). The DNA from interaction trap positive colonies can be analyzed by polymerase chain reaction (PCR) to streamline screening and detect redundant clones in cases where many positives are obtained in screening (see Alternate Protocol 1). The plasmids are isolated and characterized by a series of tests to confirm specificity of the interaction with the initial bait protein (Support Protocols 3 to 5). Those found to be specific are ready for further analysis (e.g., sequencing).

Contributed by Erica A. Golemis, Ilya Serebriiskii, Russell L. Finley, Jr., Mikhail G. Kolonin, Jeno Gyuris, and Roger Brent

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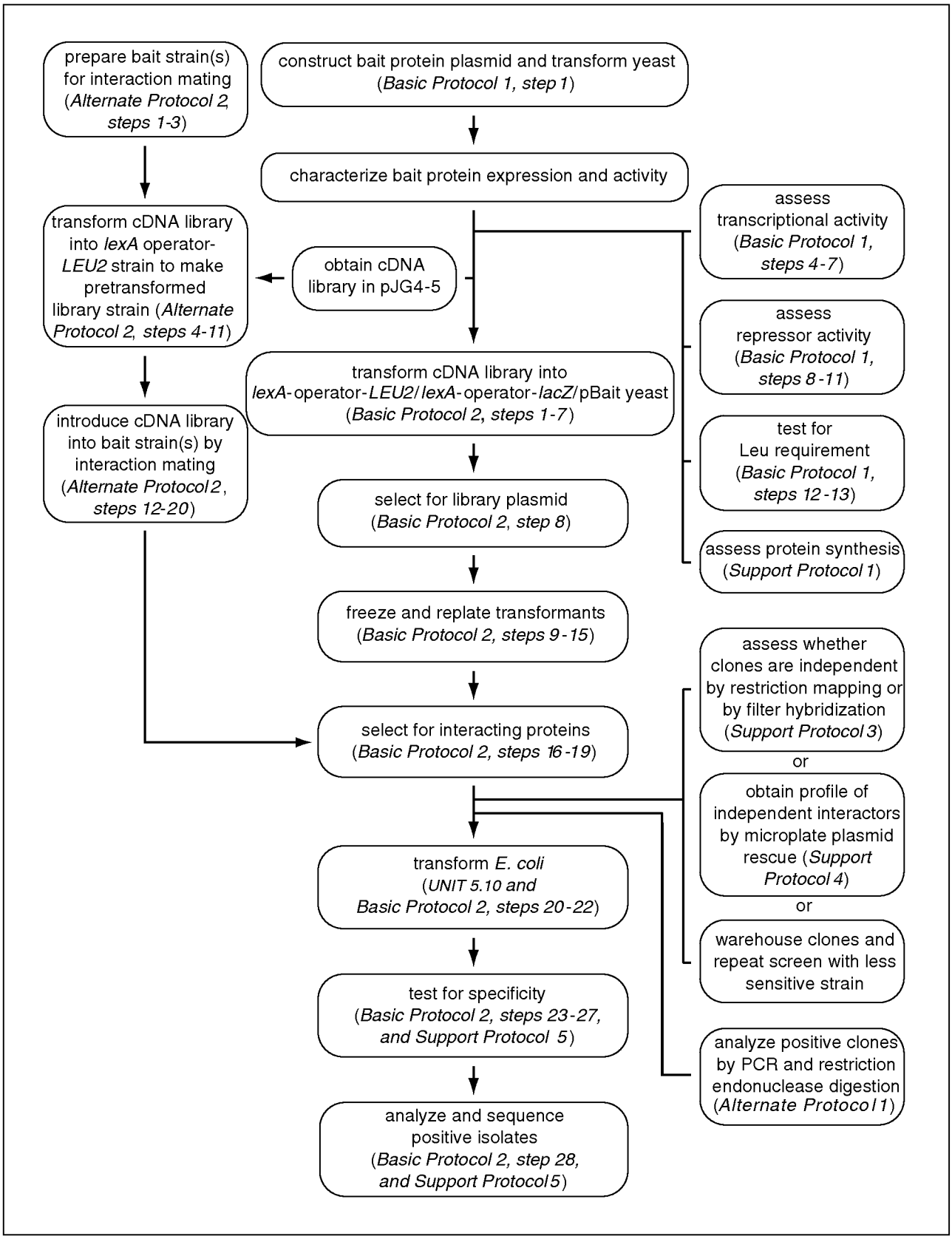

Figure 20.1.1 Flow chart for performing an interaction trap.

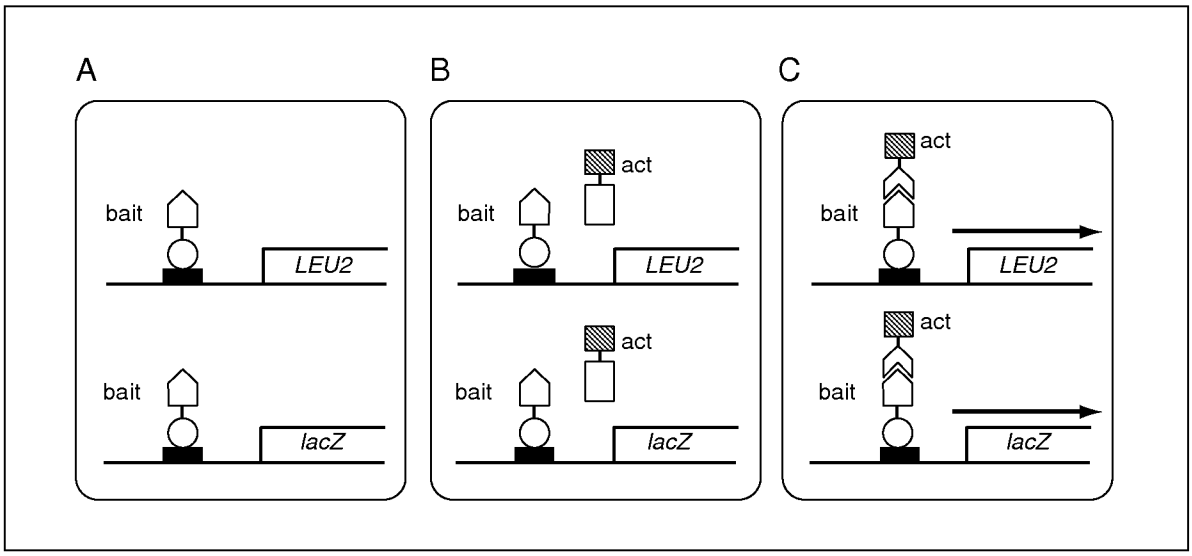

Figure 20.1.2 The interaction trap. (**A**) An EGY48 yeast cell containing two *LexA* operator–responsive reporters, one a chromosomally integrated copy of the *LEU2* gene (required for growth on –Leu medium), the second a plasmid bearing a *GAL1* promoter–*lacZ* fusion gene (causing yeast to turn blue on medium containing Xgal). The cell also contains a constitutively expressed chimeric protein, consisting of the DNA-binding domain of LexA fused to the probe or bait protein, shown as being unable to activate either of the two reporters. (**B**) and (**C**), EGY48/pSH18-34/pbait-containing yeast have been additionally transformed with an activation domain (act)–fused cDNA library in pJG4-5, and the library has been induced. In (B), the encoded protein does not interact specifically with the bait protein and the two reporters are not activated. In (C), a positive interaction is shown in which the library-encoded protein interacts with bait protein, resulting in activation of the two reporters (arrow), thus causing growth on medium lacking Leu and blue color on medium containing Xgal. Symbols: black rectangle, *LexA* operator sequence; open circle, LexA protein; open pentagon, bait protein; open rectangle, library protein; shaded box, activator protein (acid blob in Fig. 20.1.6).

When more than one bait will be used to screen a single library, significant time and resources can be saved by performing the interactor hunt by interaction mating (see Alternate Protocol 2). In this protocol, EGY48 is transformed with library DNA and the transformants are collected and frozen in aliquots. For each interactor hunt, an aliquot of the pretransformed EGY48/library strain is thawed and mixed with an aliquot of a bait strain transformed with the bait expression plasmid and pSH18-34. Overnight incubation of the mixture on a YPD plate results in fusion of the two strains to form diploids. The diploids are then exposed to galactose to induce expression of the library-encoded proteins, and interactors are selected in the same manner as in Basic Protocol 2. The advantage to this approach is that it requires only one high-efficiency library transformation for multiple hunts with different baits. It is also useful for bait proteins that are somewhat toxic to yeast; yeast expressing toxic baits can be difficult to transform with the library DNA.

CHARACTERIZING A BAIT PROTEIN

BASIC PROTOCOL 1

The first step in an interactor hunt is to construct a plasmid that expresses LexA fused to the protein of interest. This construct is transformed into reporter yeast strains containing *LEU2* and *lacZ* reporter genes, and a series of control experiments is performed to establish whether the construct is suitable as is or must be modified, and whether alternative yeast reporter conditions should be used. These controls establish that the bait protein is made as a stable protein in yeast, that it is capable of entering the nucleus and binding *LexA* operator sites, and that it does not appreciably activate transcription of the *LexA* operator–based reporter genes. This last is the most important constraint on use of this system. The LexA-fused bait protein must not activate transcription of either re-

Table 20.1.1 Interaction Trap Components[a,b]

Plasmid name/source	Selection		Comment/description
	In yeast	In *E. coli*	
LexA fusion plasmids			
pEG202[c,d,e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA followed by polylinker
pJK202	*HIS3*	Ap^r	Like pEG202, but incorporates nuclear localization sequences between LexA and polylinker; used to enhance translocation of bait to nucleus
pNLexA[e]	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses polylinker followed by LexA; for use with baits where amino-terminal residues must remain unblocked
pGilda[d]	*HIS3*	Ap^r	Contains a *GAL1* promoter that expresses same LexA and polylinker cassette as pEG202; for use with baits whose continuous presence is toxic to yeast
pEE202I	*HIS3*	Ap^r	An integrating form of pEG202 that can be targeted into *HIS3* following digestion with *Kpn*I; for use where physiological screen requires lower levels of bait to be expressed
pRFHM1[e,f] (control)	*HIS3*	Ap^r	Contains an *ADH* promoter that expresses LexA fused to the homeodomain of bicoid to produce nonactivating fusion; used as positive control for repression assay, negative control for activation and interaction assays
pSH17-4[e,f] (control)	*HIS3*	Ap^r	*ADH* promoter expresses LexA fused to GAL4 activation domain; used as a positive control for transcriptional activation
pMW101[f]	*HIS3*	Cm^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pMW103[f]	*HIS3*	Km^r	Same as pEG202, but with altered antibiotic resistance markers; basic plasmid used for cloning bait
pHybLex/Zeo[f,g]	Zeo^r	Zeo^r	Bait cloning vector compatible with interaction trap and all other two-hybrid systems; minimal ADH promotor expresses LexA followed by extended polylinker
Activation domain fusion plasmids			
pJG4-5[c,d,e,f]	TRP1	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, HA epitope tag, cloning sites; used to express cDNA libraries
pJG4-5I	*TRP1*	Ap^r	An integrating form of pJG4-5 that can be targeted into *TRP1* by digestion with *Bsu*36I (New England Biolabs); to be used with pEE202I to study interactions that occur physiologically at low protein concentrations
pYESTrp[g]	*TRP1*	Ap^r	Contains a *GAL1* promoter that expresses nuclear localization domain, transcriptional activation domain, V5 epitope tag, multiple cloning sites; contains f1 ori and T7 promoter/flanking site; used to express cDNA libraries (Invitrogen)
pMW102[f]	*TRP1*	Km^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
pMW104[f]	*TRP1*	Cm^r	Same as pJG4-5, but with altered antibiotic resistance markers; no libraries yet available
LacZ reporter plasmids			
pSH18-34[d,e,f]	*URA3*	Ap^r	Contains 8 *LexA* operators that direct transcription of the *lacZ* gene; one of the most sensitive indicator plasmids for transcriptional activation
pJK103[e]	*URA3*	Ap^r	Contains two *LexA* operators that direct transcription of the *lacZ* gene; an intermediate reporter plasmid for transcriptional activation
pRB1840[e]	*URA3*	Ap^r	Contains 1 *LexA* operator that directs transcription of the *lacZ* gene; one of the most stringent reporters for transcriptional activation
pMW112[f]	*URA3*	Km^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW109[f]	*URA3*	Km^r	Same as pJK103, but with altered antibiotic resistance marker

continued

Table 20.1.1 Interaction Trap Components[a,b], continued

Plasmid name/source	Selection		Comment/description
	In yeast	In *E. coli*	
pMW111[f]	*URA3*	Km^r	Same as pRB1840, but with altered antibiotic resistance marker
pMW107[f]	*URA3*	Cm^r	Same as pSH18-34, but with altered antibiotic resistance marker
pMW108[f]	*URA3*	Cm^r	Same as pJK103, but with altered antibiotic resistance marker
pMW110[f]	*URA3*	Cm^r	Same as pRB1840, but with altered antibiotic resistance marker
pJK101[e,f] (control)	*URA3*	Ap^r	Contains a *GAL1* upstream activating sequence followed by two *lexA* operators followed by *lacZ* gene; used in repression assay to assess bait binding to operator sequences

[a]All plasmids contain a 2μm origin for maintenance in yeast, as well as a bacterial origin of replication, except where noted (pEE202I, pJG4.5I).

[b]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Plasmids with altered antibiotic resistance markers (all pMW plasmids) were constructed at Glaxo in Research Triangle Park, N.C. (Watson et al., 1996). Plasmids and strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (pEG202); J. Kamens, BASF, Worcester, Mass. (pJK202); cumulative efforts of I. York, Dana-Farber Cancer Center, Boston, Mass. and M. Sainz and S. Nottwehr, U. Oregon (pNLexA); D.A. Shaywitz, MIT Center for Cancer Research, Cambridge, Mass. (pGilda); R. Buckholz, Glaxo, Research Triangle Park, N.C. (pEE2021, pJG4-51); J. Gyuris, Mitotix, Cambridge, Mass. (pJG4-5); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH17-4); R.L. Finley, Wayne State University School of Medicine, Detroit, Mich. (pRFHM1); S. Hanes, Wadsworth Institute, Albany, N.Y. (pSH18-34); J. Kamens, BASF, Worcester, Mass. (pJK101, pJK103); R. Brent, The Molecular Sciences Institute, Berkeley, Calif. (pRB1840). Specialized plasmids not yet commercially available can be obtained by contacting the Brent laboratory at (510) 647-0690 or *brent@molsci.org*, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[c]Sequence data are available for pEG202 (pLexA) accession number pending.

[d]Plasmids commercially available from Clontech and OriGene; for Clontech pEG202 is listed as pLexA, pJG4-5 as pB42AD, and pSH18-34 as p8op-LacZ.

[e]Plasmids and strains available from OriGene.

[f]In pMW plasmids the ampicillin resistance gene (Ap^r) is replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r) from pBC SK(+) and pBK-CMV (Stratagene), respectively. The choice between Km^r and Cm^r or Ap^r plasmids is a matter of personal taste; use of basic Ap^r plasmids is described in the basic protocols. Use of the more recently developed reagents would facilitate the purification of library plasmid in later steps by eliminating the need for passage through KC8 bacteria, with substantial saving of time and effort. Ap^r has been maintained as marker of choice for the library plasmid because of the existence of multiple libraries already possessing this marker. These plasmids are the basic set of plasmids recommended for use.

[g]Plasmids commercially available from Invitrogen as components of a Hybrid Hunter kit; this kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

porter—the EGY48 strain (or related strain EGY191) that expresses the LexA fusion protein should not grow on medium lacking Leu, and the colonies should be white on medium containing Xgal. The characterized bait protein plasmid is used for Basic Protocol 2 to screen a library for interacting proteins.

Materials

DNA encoding the protein of interest

Plasmids (see Table 20.1.1): pEG202 (see Fig. 20.1.3), pSH18-34 (see Fig. 20.1.4), pSH17-4, pRFHM1, and pJK101 for basic characterization; other plasmids for specific circumstances as described (Clontech, Invitrogen, OriGene, or R. Brent)

Yeast strain EGY48 (*ura3 trp1 his3 3LexA*-operator-*LEU2*), or EGY191 (*ura3 trp1 his3 1LexA*-operator-*LEU2*; Table 20.1.2)

Complete minimal (CM) medium dropout plates (*UNIT 13.1*), supplemented with 2% (w/v) of the indicated sugars (glucose or galactose), in 100-mm plates:
- Glu/CM, –Ura, –His
- Gal/CM, –Ura, –His
- Gal/CM, –Ura, –His, –Leu

Z buffer (*UNIT.13.6*) with 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (Xgal)

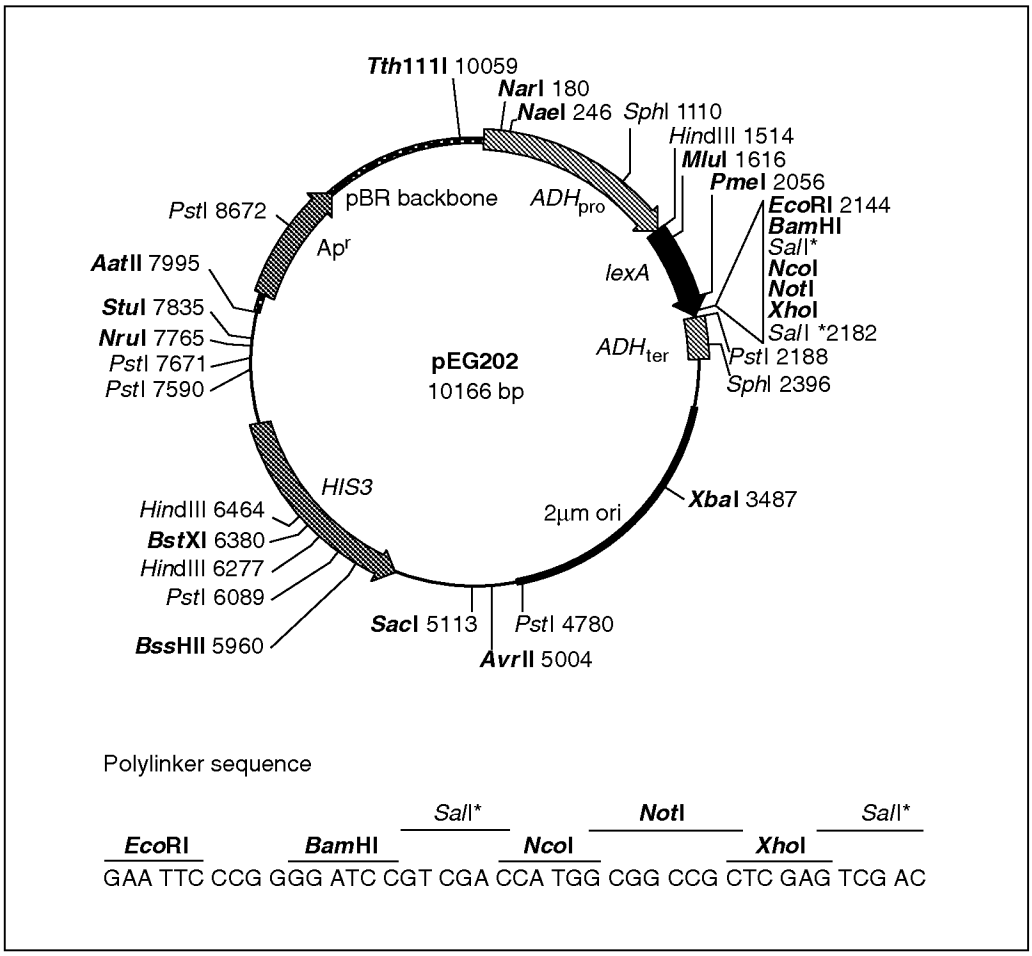

Figure 20.1.3 LexA-fusion plasmids: pEG202. The strong constitutive *ADH* promoter is used to express bait proteins as fusions to the DNA-binding protein LexA. Restriction sites shown in this map are based on recently compiled pEG202 sequence data and include selected sites suitable for diagnostic restriction endonuclease digests. A number of restriction sites are available for insertion of coding sequences to produce protein fusions with LexA; the polylinker sequence and reading frame relative to LexA are shown below the map with unique sites marked in bold type. The sequence 5′-CGT CAG CAG AGC TTC ACC ATT G-3′ can be used to design a primer to confirm correct reading frame for LexA fusions. Plasmids contain the *HIS3* selectable marker and the 2μm origin of replication to allow propagation in yeast, and the Ap^r antibiotic resistance gene and the pBR origin of replication to allow propagation in *E. coli*. In the recently developed LexA-expression plasmids pMW101 and pMW103, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively (see Table 19.2.1 for details).

Gal/CM dropout liquid medium (*UNIT 13.1*) supplemented with 2% Gal

Antibody to LexA or fusion domain: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

H_2O, sterile

30°C incubator

Nylon membrane

Whatman 3MM filter paper

Additional reagents and equipment for subcloning DNA fragments (*UNIT 3.16*), lithium acetate transformation of yeast (*UNIT 13.7*), liquid assay for β-galactosidase (*UNIT 13.6*), preparation of protein extracts for immunoblot analysis (see Support Protocol 1), and immunoblotting and immunodetection (*UNIT 10.8*)

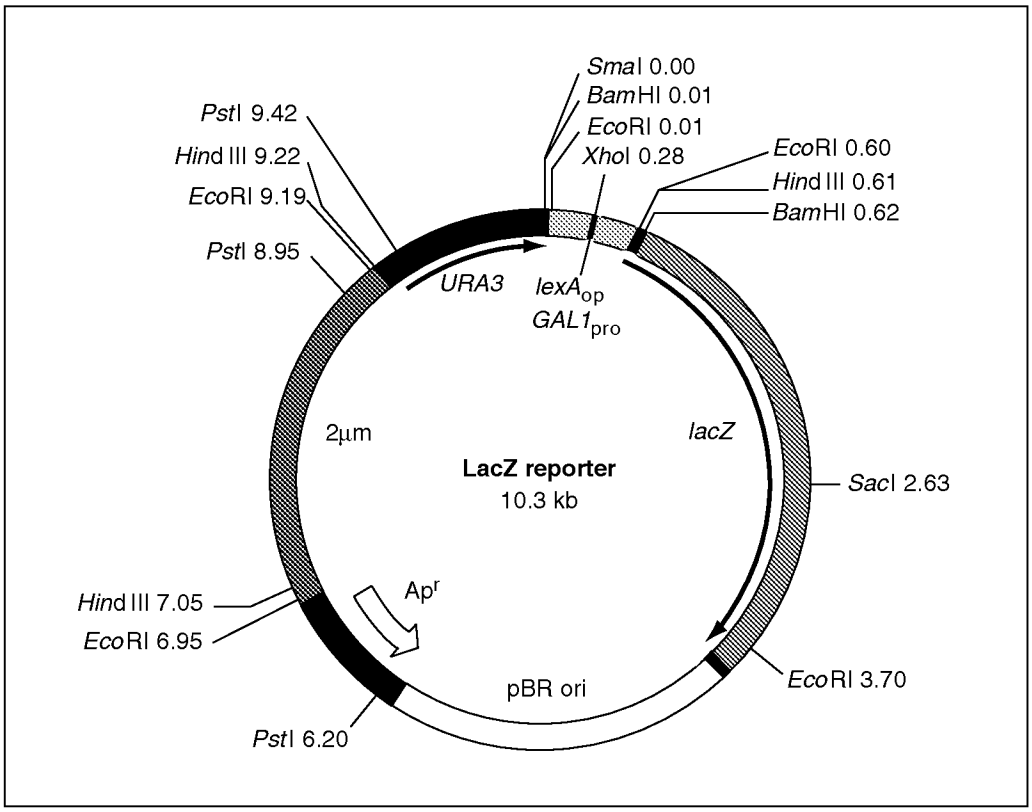

Figure 20.1.4 LacZ reporter plasmid. pRB1840, pJK103, and pSH18-34 are all derivatives of LR1Δ1 (West et al., 1984) containing eight, two, or one operator for LexA (*LexA*$_{op}$) binding inserted into the unique *Xho*I site located in the minimal *GAL1* promoter (*GAL1*$_{pro}$; 0.28 on map). The plasmid contains the *URA3* selectable marker, the 2μm origin to allow propagation in yeast, the ampicillin resistance (Ap^r) gene, and the pBR322 origin (ori) to allow propagation in *E. coli*. Numbers indicate relative map positions. In the recently developed derivatives, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol or kanamycin resistance genes (see Table 19.2.1 for details).

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

Transform yeast with the bait protein plasmid

1. Using standard subcloning techniques (*UNIT 3.16*), insert the DNA encoding the protein of interest into the polylinker of pEG202 (see Fig. 20.1.3) or other LexA fusion plasmid to make an in-frame protein fusion.

   The LexA fusion protein is expressed from the strong alcohol dehydrogenase (ADH) promoter. pEG202 also contains a HIS3 selectable marker and a 2μm origin for propagation in yeast. pEG202 with the DNA encoding the protein of interest inserted is designated pBait. Uses of alternative LexA fusion plasmids are described in Background Information.

2. Perform three separate lithium acetate transformations (*UNIT 13.7*) of EGY48 using the following combinations of plasmids:

   pBait + pSH18-34 (test)
   pSH17-4 + pSH18-34 (positive control for activation)
   pRFHM1 + pSH18-34 (negative control for activation).

   Use of the two LexA fusions as positive and negative controls allows a rough assessment of the transcriptional activation profile of LexA bait proteins. pEG202 itself is not a good negative control because the peptide encoded by the uninterrupted polylinker sequences is itself capable of very weakly activating transcription.

Table 20.1.2 Interaction Trap Yeast Selection Strains[a]

Strain	Relevant genotype	Number of operators	Comments/description
EGY48[b,c,d]	*MAT*α *trp1*, *his3*, *ura3*, lexAops-LEU2	6	*lexA* operators direct transcription from the *LEU2* gene; EGY48 is a basic strain used to select for interacting clones from a cDNA library
EGY191	*MAT*α *trp1*, *his3*, *ura3*, lexAops-LEU2	2	EGY191 provides a more stringent selection than EGY48, producing lower background with baits with instrinsic ability to activate transcription
L40[c]	MATα *trpl*, *leu2*, *ade2*, GAL4, *lexAops-HIS34*, *lexAops-lacZ8*		Expression driven from *GAL1* promoter is constitutive in L40 (inducible in EGY strains); selection is for *HIS* prototrophy. Integrated *lacZ* reporter is considerably less sensitive than pSH18-34 maintained in EGY strains

[a]Interaction Trap reagents represent the work of many contributors: the original basic reagents were developed in the Brent laboratory (Gyuris et al., 1993). Strains for specialized applications have been developed by the following individuals: E. Golemis, Fox Chase Cancer Center, Philadelphia, Pa. (EGY48, EGY191); A.B. Vojtek and S.M. Hollenberg, Fred Hutchinson Cancer Research Center, Seattle, Wash. (L40). Specialized strains not yet commercially available can be obtained by contacting the Brent laboratory at The Molecular Sciences Institute, Berkeley, (510) 647-0690 or *brent@molsci.org*, or the Golemis laboratory, (215) 728-2860 or *EA_Golemis@fccc.edu*.

[b]Strains commercially available from Clontech.

[c]Strains commercially available from Invitrogen as components of a Hybrid Hunter kit; the kit also includes all necessary positive and negative controls (not listed in this table). See Background Information for further details on commercially available reagents.

[d]Strains commercially available from OriGene.

pSH18-34 contains a 2μm origin and a URA3 selectable marker for maintenance in yeast, as well as a bacterial origin of replication and ampicillin-resistance gene. It is the most sensitive lacZ reporter available and will detect any potential ability to activate lacZ transcription. pSH17-4 is a HIS3 2μm plasmid encoding LexA fused to the activation domain of the yeast activator protein GAL4. This fusion protein strongly activates transcription. pRFHM1 is a HIS3 2μm plasmid encoding LexA fused to the N-terminus of the Drosophila protein bicoid. This fusion protein has no ability to activate transcription.

3. Plate each transformation mixture on Glu/CM –Ura, –His dropout plates. Incubate 2 days at 30°C to select for yeast that contain both plasmids.

   Colonies obtained can be used simultaneously in tests for the activation of lacZ (steps 4 to 7) and LEU2 (steps 12 to 13) reporters.

Assay lacZ gene activation by β-galactosidase assay

4. Streak a Glu/CM –Ura, –His master dropout plate with at least five or six independent colonies obtained from each of the three transformations in step 3 (test, positive control, and negative control) and incubate overnight at 30°C.

   The filter assay described in Steps 5a to 7a (based on Breeden and Nasmyth, 1985) provides a rapid assay for β-galactosidase transcription. Alternatively, a liquid assay (UNIT 13.6) or a plate assay (described in Steps 5b to 7b) may be used.

Perform filter assay for β-galactosidase activity:

5a. Lift colonies by gently placing a nylon membrane on the yeast plate and allowing it to become wet through. Remove the membrane and air dry 5 min. Chill the membrane, colony side up, 10 min at –70°C.

   Whatman 3MM filters can be cut to the size of the yeast plate as a more economical alternative to nylon membranes for performing lifts. In addition, two or three 5-min temperature cycles (–70°C to room temperature) can be used instead of a single cycle to promote better lysis; this may be worth doing if there is difficulty visualizing blue color.

6a. Cut a piece of Whatman 3MM filter paper slightly larger than the colony membrane and soak it in Z buffer containing 1 mg/ml Xgal. Place colony membrane, colony side up, on Whatman 3MM paper, or float it in the lid of a petri dish containing ~2 ml Z buffer with 1 mg/ml Xgal.

Acceptable results may be obtained using as little as 300 μg/ml Xgal.

7a. Incubate at 30°C and monitor for color changes.

It is generally useful to check the membrane after 20 min, and again after 2 to 3 hr. Strong activators will produce a blue color in 5 to 10 min, and a bait protein (LexA fusion protein) that does so is unsuitable for use in an interactor hunt using this lacZ reporter plasmid. Weak activators will produce a blue color in 1 to 6 hr (compare versus negative control pRFHMI which will itself produce a faint blue color with time) and may or may not be suitable. Weak activators should be tested using the repressor assay described in steps 8 to 11.

Perform Xgal plate assay for lacZ activation:

5b. Prepare Z buffer Xgal plates as described in UNIT 13.1.

For activation assays, plates should be prepared with glucose as a sugar source. For repression assays (steps 8 to 11), galactose should be used as a sugar source. In our experience, when patching from a master plate to Xgal plates, sufficient yeast are transferred that plasmid loss is not a major problem even in the absence of selection; this is balanced by the desire to assay sets of constructs on the same plate to eliminate batch variation in Xgal potency. Hence, plates should be made either with complete minimal amino acid mix, or by dropping out only uracil (–Ura), to make the plates universally useful.

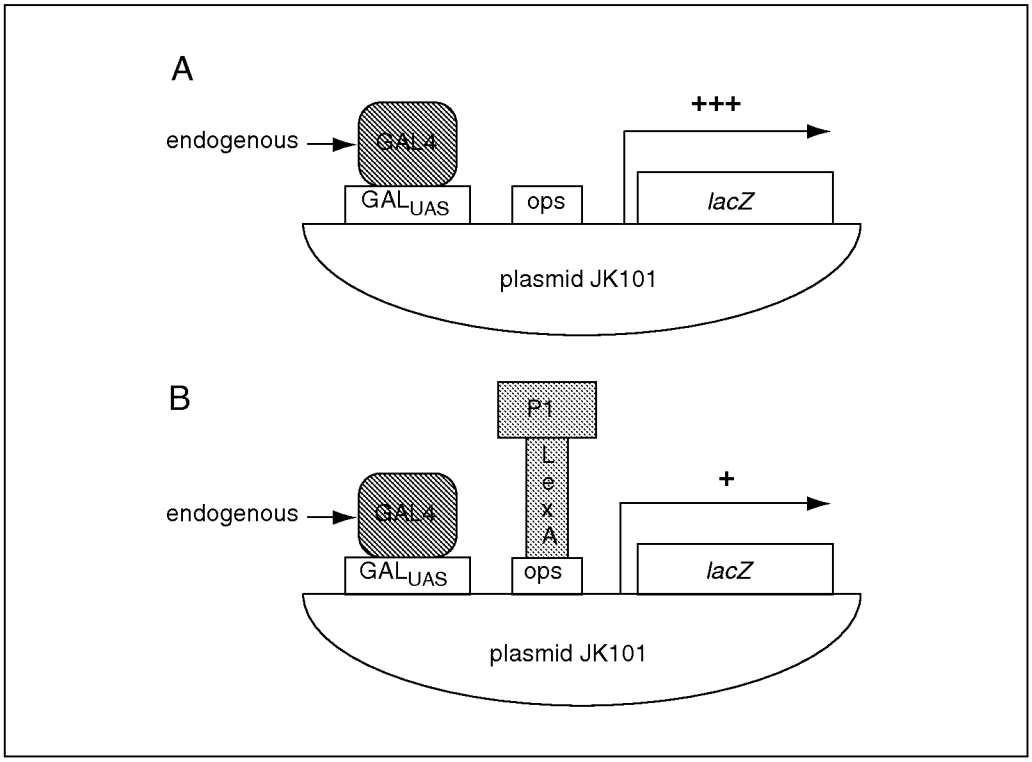

Figure 20.1.5 Repression assay for DNA binding. (**A**) The plasmid JK101 contains the upstream activating sequence (UAS) from the *GAL1* gene followed by *LexA* operators upstream of the *lacZ* coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS} (**B**). LexA-fused proteins (P1-LexA) that are made, enter the nucleus, and bind the *LexA* operator sequences (ops) will block activation from the GAL_{UAS}, repressing β-galactosidase activity (+) 3- to 5-fold. On glucose/Xgal medium, yeast containing pJK101 should be white because GAL_{UAS} transcription is repressed.

6b. Streak yeast from master plate to Xgal plate and incubate at 30°C.

7b. Examine plates for color development at intervals over the next 2 to 3 days.

Strongly activating fusions should be visibly blue on the plate within 12 to 24 hr; moderate activators will be visibly blue after ~2 days.

When a bait protein appreciably activates transcription under these conditions, there are several recourses. The first and simplest is to switch to a less sensitive lacZ reporter plasmid; use of pJK103 and pRB1840 may be sufficient to reduce background to manageable levels. If this fails to work, it is frequently possible to generate a truncated LexA fusion that does not activate transcription.

Confirm fusion-protein synthesis by repression assay

For LexA fusions that do not activate transcription, confirm by performing a repression assay (Brent and Ptashne, 1984) that the LexA fusion protein is being synthesized in yeast (some proteins are not) and that it is capable of binding *LexA* operator sequences (Fig. 20.1.5). The following steps can be performed concurrently with the activation assay.

8. Transform EGY48 yeast with the following combinations of plasmids (three transformations):

   pBait + pJK101 (test)
   pRFHM1 + pJK101 (positive control for repression)
   pJK101 alone (negative control for repression).

9. Plate each transformation mix on Glu/CM –Ura, –His dropout plates or Glu/CM –Ura dropout plates as appropriate to select yeast cells that contain the indicated plasmids. Incubate 2 to 3 days at 30°C until colonies appear.

10. Streak colonies to a Glu/CM –Ura, –His or Glu/CM –Ura dropout master plate and incubate overnight at 30°C.

11. Assay β-galactosidase activity of the three transformed strains (test, positive control, and negative control) by liquid assay (using Gal/CM dropout liquid medium), filter assay (steps 5a to 7a, first restreaking to Gal/CM plates to grow overnight), or plate assay (steps 5b to 7b, using Gal/CM –Ura XGal plates).

    This assay should not be run for more than 1 to 2 hr for membranes, or 36 hr for Xgal plates, as the high basal lacZ activity will make differential activation of pJK101 impossible to see with longer incubations. Use of Xgal plates, and inspection 12 to 24 hr after streaking, is generally most effective.

    The plasmid pJK101 contains the galactose upstream activating sequence (UAS) followed by LexA operators upstream of the lacZ coding sequence. Thus, yeast containing pJK101 will have significant β-galactosidase activity when grown on medium in which galactose is the sole carbon source because of binding of endogenous yeast GAL4 to the GAL_{UAS}. LexA-fused proteins that are made, enter the nucleus, and bind the LexA operator sequences block activation from the GAL_{UAS}, repressing β-galactosidase activity 3- to 20-fold. Note that on Glu/Xgal medium, yeast containing pJK101 should be white, because GAL_{UAS} transcription is repressed.

12. If a bait protein neither activates nor represses transcription, perform immunoblot analysis by probing an immunoblot of a crude lysate with antibodies against LexA or the fusion domain to test for protein synthesis (see Support Protocol 1).

    Even if a bait protein represses transcription, it is generally a good idea to assay for the production of full-length LexA fusions, as occasionally some fusion proteins will be proteolytically cleaved by endogenous yeast proteases. If the protein is made but does not repress, it may be necessary to clone the sequence into a LexA fusion vector that contains a nuclear localization motif, e.g., pJK202 (see Table 20.1.1), or to modify or truncate the fusion domain to remove motifs that target it to other cellular compartments (e.g., myristoylation signals).

Test for Leu requirement

These steps can be performed concurrently with the *lacZ* activation and repression assays.

13. Disperse a colony of EGY48 containing pBait and pSH18-34 reporter plasmids into 500 µl sterile water. Dilute 100 µl of suspension into 1 ml sterile water. Make a series of 1/10 dilutions in sterile water to cover a 1000-fold concentration range.

14. Plate 100 µl from each tube (undiluted, 1/10, 1/100, and 1/1000) on Gal/CM –Ura, –His dropout plates and on Gal/CM –Ura, –His, –Leu dropout plates. Incubate overnight at 30°C.

    There will be a total of eight plates. Gal/CM –Ura, –His dropout plates should show a concentration range from 10 to 10,000 colonies and Gal/CM –Ura, –His, –Leu dropout plates should have no colonies.

    Actual selection in the interactor hunt is based on the ability of the bait protein and acid-fusion pair, but not the bait protein alone, to activate transcription of the LexA operator-LEU2 gene and allow growth on medium lacking Leu. Thus, the test for the Leu requirement is the most important test of whether the bait protein is likely to have an unworkably high background. The LEU2 reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits, so it is possible that a bait protein that gives little or no signal in a β-galactosidase assay would nevertheless permit some level of growth on –Leu medium. If this occurs, there are several options for proceeding, the most immediate of which is to substitute EGY191 (see Table 20.1.2), a less sensitive screening strain, and repeat the assay.

    As outlined in this protocol, the authors recommend the strategy of performing the initial screening using the most sensitive reporters and then, if activation is detected, screening with increasingly less sensitive reporters (see Critical Parameters for further discussion).

PERFORMING AN INTERACTOR HUNT

BASIC PROTOCOL 2

An interactor hunt involves two successive large platings of yeast containing LexA-fused probes and reporters and libraries in pJG4-5 (Fig. 20.1.6, Table 20.1.3) with a cDNA expression cassette under control of the *GAL* promoter. In the first plating, yeast are plated on complete minimal (CM) medium –Ura, –His, –Trp dropout plates with glucose (Glu) as a sugar source to select for the library plasmid. In the second plating, which selects for yeast that contain interacting proteins, a slurry of primary transformants is plated on CM –Ura, –His, –Trp, –Leu dropout plates with galactose/raffinose (Gal/Raff) as the sugar source. This two-step selection is encouraged for two reasons. First, a number of interesting cDNA-encoded proteins may be deleterious to the growth of yeast that bear them; these would be competed out in an initial mass plating. Second, it seems likely that immediately after simultaneous transformation and Gal induction, yeast bearing particular interacting proteins may not be able to initially express sufficient levels of these proteins to support growth on medium lacking Leu. Library plasmids from colonies identified in the second plating are purified by bacterial transformation and used to transform yeast cells for the final specificity screen.

A list of libraries currently available for use with this system is provided in Table 20.1.3. The protocol outlined below describes the steps used to perform a single-step screen that should saturate a library derived from a mammalian cell. For screens with libraries derived from lower eukaryotes with less complex genomes, fewer plates will be required.

Occasionally, baits that seemed well-behaved during preliminary tests produce unworkably high backgrounds of "positives" during an actual screen (see Background Information and Critical Parameters). To forestall the waste of time and materials performing a screen with such a bait would entail, an alternative approach is to perform a scaled-back

screen when working with a new bait (e.g., 5 rather than 30 plates of primary transformants). The results can be assessed before doing a full screen; it is then possible to switch to lower-sensitivity reporter strains and plasmids, if appropriate. Although individual baits will vary, the authors' current default preference is to use the *lacZ* reporter pJK103 in conjunction with either EGY48 or EGY191. Polymerase chain reaction (PCR) can also be used in a rapid screening approach that may be preferable if a large number of positions are obtained in a library screen (see Alternate Protocol 1).

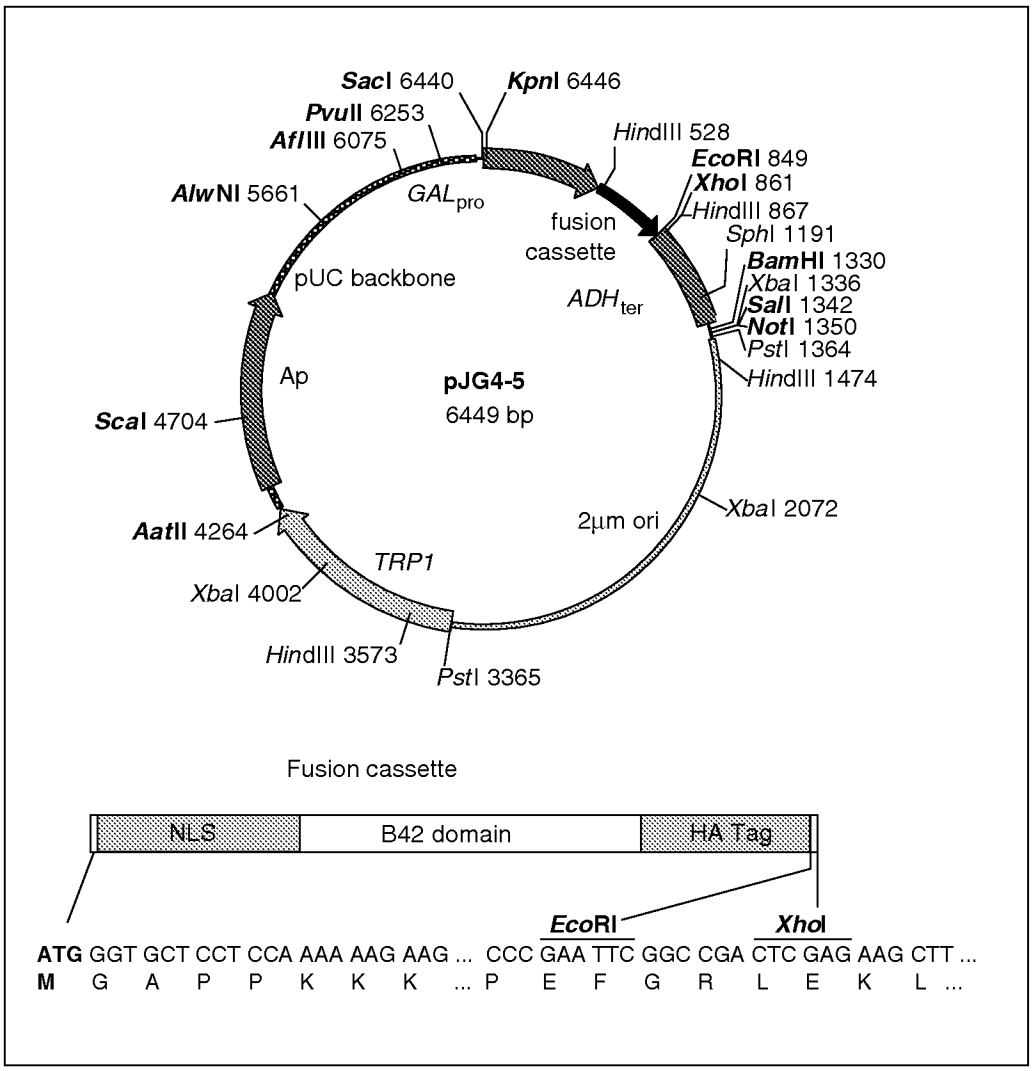

Figure 20.1.6 Library plasmids: pJG4-5. Library plasmids express cDNAs or other coding sequences inserted into unique *Eco*RI and *Xho*I sites as a translational fusion to a cassette consisting of the SV40 nuclear localization sequence (NLS; PPKKKRKVA), the acid blob B42 domain (Ruden et al, 1991), and the hemagglutinin (HA) epitope tag (YPYDVPDYA). Expression of cassette sequences is under the control of the *GAL1* galactose-inducible promoter. This map is based on the sequence data available for pJG4-5, and includes selected sites suitable for diagnostic restriction digests (shown in bold). The sequence 5′-CTG AGT GGA GAT GCC TCC-3′ can be used as a primer to identify inserts or to confirm correct reading frame. The pJG4-5 plasmid contains the *TRP1* selectable marker and the 2µm origin to allow propagation in yeast, and the antibiotic resistance gene and the pUC origin to allow propagation in *E. coli*. In the recently developed pJG4-5 derivative plasmids pMW104 and pMW102, the ampicillin resistance gene (Ap^r) has been replaced with the chloramphenicol resistance gene (Cm^r) and the kanamycin resistance gene (Km^r), respectively (see Table 19.2.2 for details). Currently existing libraries are all made in the pJG4-5 plasmid (Gyuris et al., 1993) shown on this figure. Unique sites are marked in bold type.

Materials

Yeast containing appropriate combinations of plasmids (see Table 20.1.1 and Table 20.1.2):
- EGY48 containing *LexA*-operator-*lacZ* reporter and pBait (see Basic Protocol 1)
- EGY48 containing *LexA*-operator-*lacZ* reporter and pRFHM-1
- EGY48 containing *LexA*-operator-*lacZ* reporter and any nonspecific bait

Complete minimal (CM) dropout liquid medium (*UNIT 13.1*) supplemented with sugars (glucose, galactose, and/or raffinose) as indicated [2% (w/v) Glu, or 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM –Ura, –His
- Glu/CM –Trp
- Gal/Raff/CM –Ura, –His, –Trp

H_2O, sterile

TE buffer (pH 7.5; *APPENDIX 2*)/0.1 M lithium acetate

Library DNA in pJG4-5 (Table 20.1.3 and Fig. 20.1.6)

High-quality sheared salmon sperm DNA (see Support Protocol 2)

40% (w/v) polyethylene glycol 4000 (PEG 4000; filter sterilized)/0.1 M lithium acetate/TE buffer (pH 7.5)

Dimethyl sulfoxide (DMSO)

Complete minimal (CM) medium dropout plates (*UNIT 13.1*) supplemented with sugars and Xgal (20 µg/ml) as indicated [2% (w/v) Glu, and 2% (w/v) Gal + 1% (w/v) Raff]:
- Glu/CM –Ura, –His, –Trp, 24 × 24–cm (Nunc) and 100-mm
- Gal/Raff/CM –Ura, –His, –Trp, 100-mm
- Gal/Raff/CM –Ura, –His, –Trp, –Leu, 100-mm
- Glu/Xgal/CM –Ura, –His, –Trp, 100-mm
- Gal/Raff/Xgal/CM –Ura, –His, –Trp, 100-mm
- Glu/CM –Ura, –His, –Trp, –Leu, 100-mm
- Glu/CM –Ura, –His, 100-mm
- Gal/CM –Ura, –His, –Trp, –Leu, 100-mm

TE buffer (pH 7.5), sterile (optional)

Glycerol solution (see recipe)

E. coli KC8 (*pyrF leuB600 trpC hisB463*; constructed by K. Struhl and available from R. Brent)

LB/ampicillin plates (*UNIT 1.1*)

E. coli DH5α or other strain suitable for preparation of DNA for sequencing

Bacterial defined minimal A medium plates: 1× A medium plates containing 0.5 µg/ml vitamin B1 (*UNIT 1.1*) and supplemented with 40 µg/ml each Ura, His, and Leu

30°C incubator, with and without shaking

Low-speed centrifuge and rotor

50-ml conical tubes, sterile

1.5-ml microcentrifuge tubes, sterile

42°C heating block

Glass microscope slides, sterile

Additional reagents and equipment for rapid miniprep isolation of yeast DNA (*UNIT 13.11*), transformation of bacteria by electroporation (*UNIT 1.8*), miniprep isolation of bacterial DNA (*UNIT 1.6*), restriction endonuclease digestion (*UNIT 3.1*; optional), and agarose gel electrophoresis (*UNIT 2.5A*; optional)

NOTE: All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

Table 20.1.3 Libraries Compatible with the Interaction Trap System[a]

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Cell lines				
HeLa cells (human cervical carcinoma)	JG	9.6×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
HeLa cells (human cervical carcinoma)	Y	3.7×10^6	0.3-1.2 kb	Invitrogen
WI-38 cells (human lung fibroblasts), serum-starved, cDNA	JG	5.7×10^6	0.3-3.5 kb (1.5 kb)	R. Brent, Clontech, OriGene
Jurkat cells (human T cell leukemia), exponentially growing, cDNA	JG	4.0×10^6	0.7-2.8 kb (1.5 kb)	R. Brent
Jurkat cells (human T cell leukemia)	Y	3.2×10^6	0.3-1.2 kb	Invitrogen
Jurkat cells (human T cell leukemia)	Y	3.0×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Jurkat cells (human T cell leukemia)	JG	5.7×10^6	(>1.3)	OriGene
Jurkat cells (human T cell leukemia)	JG	2×10^6	0.7-3.5 kb (1.2 kb)	S. Witte
Be Wo cells (human fetal placental choriocarcinoma)	Y	5.4×10^6	0.3-0.8 kb	Invitrogen
Human lymphocyte	JG	4.0×10^6	0.4-4.0 kb (2.0 kb)	Clontech
$CD4^+$ T cell, murine, cDNA	JG	$>10^6$	0.3-2.5 kb (>0.5 kb)	R. Brent
Chinese hamster ovary (CHO) cells, exponentially growing, cDNA	JG	1.5×10^6	0.3-3.5 kb	R. Brent
A20 cells (mouse B cell lymphoma)	Y	3.11×10^6	0.3-1.2 kb	Invitrogen
Human B cell lymphoma	JG	—	—	H. Niu
Human 293 adenovirus–infected (early and late stages)	JG	—	—	K. Gustin
SKOV3 human Y ovarian cancer	Y	5.0×10^6	(>1.4 kb)	OriGene
MDBK cell, bovine kidney	JG	5.8×10^6	(>1.2 kb)	OriGene
MDCK cells	JG	—	—	D. Chen
HepG2 cell line cDNA	JG	2×10^6	—	M. Melegari
MCF7 breast cancer cells, untreated	JG	1.0×10^7	(>1.5 kb)	OriGene
MCF7 breast cancer cells, estrogen-treated	JG	1.0×10^7	(>1.1 kb)	OriGene
MCF7 cells, serum-grown	JG	1.0×10^7	0.4-3.5 kb	OriGene
LNCAP prostate cell line, untreated	JG	2.9×10^6	(>0.8 kb)	OriGene
LNCAP prostate cell line, androgen-treated	JG	4.6×10^6	(>0.9 kb)	OriGene
Mouse pachytene spermatocytes	JG	—	—	C. Hoog
Tissues				
Human breast	Y	9×10^6	0.4-1.2 kb	Invitrogen
Human breast tumor	Y	8.84×10^6	0.4-1.2 kb	Invitrogen
Human liver	JG	$>10^6$	0.6-4.0 kb (>1 kb)	R. Brent
Human liver	Y	2.2×10^6	0.5-4 kb (1.3 kb)	Clontech
Human liver	JG	3.2×10^6	0.3-1.2 kb	Invitrogen
Human liver	JG	1.1×10^7	(> 1 kb)	OriGene
Human lung	Y	5.9×10^6	0.4-1.2 kb	Invitrogen
Human lung tumor	Y	1.9×10^6	0.4-1.2	Invitrogen
Human brain	JG	3.5×10^6	0.5-4.5 kb (1.4 kb)	Clontech
Human brain	Y	8.9×10^6	0.3-1.2 kb	Invitrogen
Human testis	Y	6.4×10^6	0.3-1.2 kb	Invitrogen
Human testis	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human ovary	Y	4.6×10^6	0.3-1.2 kb	Invitrogen

continued

Table 20.1.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Human ovary	JG	4.6×10^6	(>1.3 kb)	OriGene
Human ovary	JG	3.5×10^6	0.5-4.0 kb (1.8 kb)	Clontech
Human heart	JG	3.0×10^6	0.3-3.5 kb (1.3 kb)	Clontech
Human placenta	Y	4.8×10^6	0.3-1.2 kb	Invitrogen
Human placenta	JG	3.5×10^6	0.3-4.0 kb (1.2 kb)	Clontech
Human mammary gland	JG	3.5×10^6	0.5-5 kb (1.6 kb)	Clontech
Human peripheral blood leucocyte	JG	1.0×10^7	(>1.3 kb)	OriGene
Human kidney	JG	3.5×10^6	0.4-4.5 kb (1.6 kb)	Clontech
Human fetal kidney	JG	3.0×10^6	(>1 kb)	OriGene
Human spleen	Y	1.14×10^7	0.4-1.2 kb	Invitrogen
Human prostate	Y	5.5×10^6	0.4-1.2 kb	Invitrogen
Human normal prostate	JG	1.4×10^6	0.4-4.5 kb (1.7 kb)	Clontech
Human prostate	JG	1.4×10^6	(>1 kb)	OriGene
Human prostate cancer	JG	1.1×10^6	(>0.9 kb)	OriGene
Human fetal prostate	JG	—	—	OriGene
Human fetal liver	JG	3.5×10^6	0.3-4.5 kb (1.3 kb)	Clontech
Human fetal liver	Y	2.37×10^6	0.3-1.2 kb	Invitrogen
Human fetal liver	JG	8.6×10^6	(>1 kb)	OriGene
Human fetal brain	JG	3.5×10^6	0.5-1.2 kb (1.5 kb)	R. Brent, Clontech, Invitrogen, OriGene
Mouse brain	JG	6.1×10^6	(>1 kb)	OriGene
Mouse brain	JG	4.5×10^6	0.4-4.5 kb (1.2 kb)	Clontech
Mouse breast, lactating	JG	1.0×10^7	0.4-3.1 kb	OriGene
Mouse breast, involuting	JG	1.0×10^7	0.4-7.0 kb	OriGene
Mouse breast, virgin	JG	1.0×10^7	0.4-5.5 kb	OriGene
Mouse breast, 12 days pregnant	JG	6.3×10^6	0.4-5.3 kb	OriGene
Mouse skeletal muscle	JG	7.2×10^6	0.4-3.5 kb	OriGene
Rat adipocyte, 9-week-old Zucker rat	JG	1.0×10^7	0.4-5.0 kb	OriGene
Rat brain	JG	4.5×10^6	0.3-3.4 kb	OriGene
Rat brain (day 18)	JG	—	—	H. Niu
Rat testis	JG	8.0×10^6	(>1.2 kb)	OriGene
Rat thymus	JG	8.2×10^6	(>1.3 kb)	OriGene
Mouse liver	JG	9.5×10^6	(>1.4 kb)	OriGene
Mouse spleen	JG	1.0×10^7	(>1 kb)	OriGene
Mouse ovary	JG	4.0×10^6	(>1.2 kb)	OriGene
Mouse prostate	JG	—	—	OriGene
Mouse embryo, whole (19-day)	JG	1.0×10^5	0.2-2.5 kb	OriGene
Mouse embryo	JG	3.6×10^6	0.5-5 kb (1.7 kb)	Clontech
Drosophila melanogaster, adult, cDNA	JG	1.8×10^6	(>1.0 kb)	OriGene
D. melanogaster, embryo, cDNA	JG	3.0×10^6	0.5-3.0 kb (1.4 kb)	Clontech
D. melanogaster, 0-12 hr embryos, cDNA	JG	4.2×10^6	0.5-2.5 kb (1.0 kb)	R. Brent
D. melanogaster, ovary, cDNA	JG	3.2×10^6	0.3-1.5 kb (800 bp)	R. Brent
D. melanogaster, disc, cDNA	JG	4.0×10^6	0.3-2.1 kb (900 bp)	R. Brent
D. melanogaster, head	JG	—	—	M. Rosbash

continued

Table 20.1.3 Libraries Compatible with the Interaction Trap System[a], continued

Source of RNA/DNA	Vector	Independent clones	Insert size (average)[b]	Contact information
Miscellaneous				
Synthetic aptamers	PJM-1	$>1\times 10^9$	60 bp	R. Brent
Saccharomyces cerevisiae, S288C, genomic	JG	$>3 \times 10^6$	0.8-4.0 kb	R. Brent
S. cerevisiae, S288C, genomic	JG	4.0×10^6	0.5-4.0 kb	OriGene
Sea urchin ovary	JG	3.5×10^6	(1.7 kb)	Clontech
Caenorhabditis elegans	JG	3.8×10^6	(>1.2 kb)	OriGene
Agrobacterium tumefaciens	JG	—	—	—
Arabidopsis thaliana, 7-day-old seedlings	JG	—	—	H.M. Goodman
Tomato (*Lycopersicon esculentum*)	JG	8×10^6	—	G.B. Martin
Xenopus laevis embryo	JG	2.2×10^6	0.3-4 kb (1.0 kb)	Clontech

[a]Most libraries are constructed in either the pJG4-5 vector or the pYESTrp vector (JG or Y in the Vector column); the peptide aptamer library is made in the pJM-1 vector. Libraries available from the public domain were constructed by the following individuals: (1) J. Gyuris; (3) C. Sardet and J. Gyuris; (4) W. Kolanus, J. Gyuris, and B. Seed; (39) D. Krainc; (50-52) R. Finley; (55) P. Watt; (54) P. Colas, B. Cohen, T. Jessen, I. Grishina, J. McCoy, and R. Brent (Colas et al., 1996). All libraries mentioned above were constructed in conjunction with and are available from the laboratory of Roger Brent, (510) 647-0690 or *brent@molsci.org*. The following individual investigators must be contacted directly: (18) J. Pugh, Fox Chase Cancer Center, Philadelphia, Pa.; (8,9) Vinyaka Prasad, Albert Einstein Medical Center New York, N.Y.; (57, 58) Gregory B. Martin, *gmartin@dept.agry.purdue.edu*; (11) Huifeng Niu, *hn34@columbia.edu*; (16) Christer Hoog, *christer.hoog@cmb.ki.se*; (12) Kurt Gustin, *kgus@umich.edu*; (6) Stephan Witte, *Stephan.Witte@nimbus.rz.uni-konstanz.de*.

[b]Insert size ranges for pJG4-5 based libraries originally constructed in the Brent laboratory, which are now commercially available from Clontech, were reestimated by the company.

Transform the library

1. Grow an ~20-ml culture of EGY48 or EGY191 containing a *LexA*-operator-*lacZ* reporter plasmid and pBait in Glu/CM –Ura, –His liquid dropout medium overnight at 30°C.

   For best results, the pBait and lacZ reporter plasmids should have been transformed into the yeast within ~7 to 10 days of commencing a screen.

2. In the morning, dilute culture into 300 ml Glu/CM –Ura, –His liquid dropout medium to 2×10^6 cell/ml (OD_{600} = ~0.10). Incubate at 30°C until the culture contains ~1×10^7 cells/ml (OD_{600} = ~0.50).

3. Centrifuge 5 min at 1000 to 1500 × *g* in a low-speed centrifuge at room temperature to harvest cells. Resuspend in 30 ml sterile water and transfer to 50-ml conical tube.

4. Centrifuge 5 min at 1000 to 1500 × *g*. Decant supernatant and resuspend cells in 1.5 ml TE buffer/0.1 M lithium acetate.

5. Add 1 µg library DNA in pJG4-5 and 50 µg high-quality sheared salmon sperm carrier DNA to each of 30 sterile 1.5-ml microcentrifuge tubes. Add 50 µl of the resuspended yeast solution from step 4 to each tube.

   The total volume of library and salmon sperm DNA added should be <20 µl and preferably <10 µl.

   A typical library transformation will result in 2 to 3 × 10^6 primary transformants. Assuming a transformation efficiency of 10^5/µg library DNA, this transformation requires a total of 20 to 30 µg library DNA and 1 to 2 mg carrier DNA. Doing transformations in small aliquots helps reduce the likelihood of contamination, and for reasons that are not clear, provides significantly better transformation efficiency than scaled-up versions.

   Do not use excess transforming library DNA per aliquot of competent yeast cells because each competent cell may take up multiple library plasmids, complicating subsequent analysis.

6. Add 300 µl of sterile 40% PEG 4000/0.1 M lithium acetate/TE buffer, pH 7.5, and invert to mix thoroughly. Incubate 30 min at 30°C.

7. Add DMSO to 10% (~40 µl per tube) and invert to mix. Heat shock 10 min in 42°C heating block.

8a. *For 28 tubes:* Plate the complete contents of one tube per 24 × 24–cm Glu/CM –Ura, –His, –Trp dropout plate and incubate at 30°C.

8b. *For two remaining tubes:* Plate 360 µl of each tube on 24 × 24–cm Glu/CM –Ura, –His, –Trp dropout plate. Use the remaining 40 µl from each tube to make a series of 1/10 dilutions in sterile water. Plate dilutions on 100-mm Glu/CM –Ura, –His, –Trp dropout plates. Incubate all plates 2 to 3 days at 30°C until colonies appear.

The dilution series gives an idea of the transformation efficiency and allows an accurate estimation of the number of transformants obtained.

Collect primary transformant cells

Conventional replica plating (*UNIT 13.3*) does not work well in the selection process because so many cells are transferred to new plates that very high background levels inevitably occur. Instead, the procedure described below creates a slurry in which cells derived from $>10^6$ primary transformants are homogeneously dispersed. A precalculated number of these cells is plated for each primary transformant.

9. Cool all of the 24 × 24–cm plates containing transformants for several hours at 4°C to harden agar.

10. Wearing gloves and using a sterile glass microscope slide, gently scrape yeast cells off the plate. Pool cells from the 30 plates into one or two sterile 50-ml conical tubes.

This is the step where contamination is most likely to occur. Be careful.

11. Wash cells by adding a volume of sterile TE buffer or water at least equal to the volume of the transferred cells. Centrifuge ~5 min at 1000 to 1500 × *g*, room temperature, and discard supernatant. Repeat wash.

After the second wash, pellet volume should be ~25 ml cells derived from 1.5 × 10^6 transformants.

12. Resuspend pellet in 1 vol glycerol solution, mix well, and store up to 1 year in 1-ml aliquots at –70°C.

Determine replating efficiency

13. Remove an aliquot of frozen transformed yeast and dilute 1/10 with Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 4 hr at 30°C to induce the *GAL* promoter on the library.

Raffinose (Raff) aids in growth without diminishing transcription from the GAL1 promoter.

14. Make serial dilutions of the yeast cells using the Gal/Raff/CM –Ura, –His, –Trp dropout medium. Plate on 100-mm Gal/Raff/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies are visible.

15. Count colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

In calculating yeast concentrations, it is useful to remember that 1 OD_{600} unit = ~2.0 × 10^7 yeast cells. In general, if the harvest is done carefully, viability will be greater than 90%. Some intrepid investigators perform this step simultaneously with plating out on Leu selective medium (steps 16 and 17).

Screen for interacting proteins

16. Thaw the appropriate quantity of transformed yeast based on the plating efficiency, dilute, and incubate as in step 13. Dilute cultures in Gal/Raff/CM –Ura, –His, –Trp, –Leu medium as necessary to obtain a concentration of 10^7 cells/ml (OD_{600} = ~0.5), and plate 100 µl on each of as many 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu dropout plates as are necessary for full representation of transformants. Incubate 2 to 3 days at 30°C until colonies appear.

    Because not all cells that contain interacting proteins plate at 100% efficiency on –Leu medium (Estojak et al., 1995), it is desirable that for actual selection, each primary colony obtained from the transformation be represented on the selection plate by three to ten individual yeast cells. This will in some cases lead to multiple isolations of the same cDNA; however, because the slurry is not perfectly homogenous, it will increase the likelihood that all primary transformants are represented by at least one cell on the selective plate.

    It is easiest to visually scan for Leu$^+$ colonies using cells plated at ~10^6 cfu per 100-mm plate. Plating at higher density can contribute to cross-feeding between yeast, resulting in spurious background growth. Thus, for a transformation in which 3×10^6 colonies are obtained, plate ~2×10^7 cells on a total of 20 selective plates.

17. Carefully pick appropriate colonies to a new Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate. Incubate 2 to 7 days at 30°C until colonies appear.

    A good strategy is to pick a master plate with colonies obtained on day 2, a second master plate (or set of plates) with colonies obtained on day 3, and a third with colonies obtained on day 4. Colonies from day 2 and 3 master plates should generally be characterized further. If many apparent positives are obtained, it may be worth making master plates of the much larger number of colonies likely to be obtained at day 4 (and after). See Critical Parameters and annotation to step 19 for additional information about appropriate colony selection for the master plate.

    If no colonies appear within a week, those arising at later time points are likely to be artifactual. Contamination that has occurred at an earlier step (e.g., during plate scraping) is generally reflected by the growth of a very large number of colonies (>500/plate) within 24 to 48 hr after plating on selective medium.

    Some investigators omit use of a Gal/Raff/CM –Ura, –His, –Trp, –Leu master plate, restreaking directly to a Glu/CM –Ura, –His, –Trp master plate as in step 19.

Test for Gal dependence

The following steps test for Gal dependence of the Leu$^+$ insert and lacZ phenotypes to confirm that they are attributable to expression of the library-encoded proteins. The *GAL1* promoter is turned off and –Leu selection eliminated before reinducing.

18. Restreak from the Gal/Raff/CM –Ura, –His, –Trp, –Leu master dropout plate to a 100-mm Glu/CM –Ura, –His, –Trp master dropout plate. Incubate overnight at 30°C until colonies form.

19. Restreak or replica plate from this plate to the following plates:

    Glu/Xgal/CM –Ura, –His, –Trp
    Gal/Raff/Xgal/CM –Ura, –His, –Trp
    Glu/CM –Ura, –His, –Trp, –Leu
    Gal/Raff/CM –Ura, –His, –Trp, –Leu.

    At this juncture, colonies and the library plasmids they contain are tentatively considered positive if they are blue on Gal/Raff/Xgal plates but not blue or only faintly blue on Glu/Xgal plates, and if they grow on Gal/Raff/CM –Leu plates but not on Glu/CM –Leu plates.

    The number of positives obtained will vary drastically from bait to bait. How they are processed subsequently will depend on the number initially obtained and on the preference

of the individual investigator. If none are obtained using EGY48 as reporter strain, it may be worth attempting to screen a library from an additional tissue source. If a relatively small number (≤30) are obtained, proceed to step 20. However, sometimes searches will yield large numbers of colonies (>30 to 300, or more). In this case, there are several options. The first option is to warehouse the majority of the positives and work up the first 30 that arise; those growing fastest are frequently the strongest interactors. These can be checked for specificity, and restriction digests can be used to establish whether they are all independent cDNAs or represent multiple isolates of the same, or a small number, of cDNAs. If the former is true, it may be advisable to repeat the screen in a less sensitive strain background, as obtaining many different interactors can be a sign of low-affinity nonspecific background. Alternatively, if initial indications are that a few cDNAs are dominating the positives obtained, it may be useful to perform a filter hybridization with yeast (see Support Protocol 3) using these cDNAs as a probe to establish the frequency of their identification and exclude future reisolation of these plasmids. The second major option is to work up large numbers of positives to get a complete profile of isolated interactors (see Support Protocol 4). A third option is to temporarily warehouse the entire results of this first screen, and repeat the screen with a less sensitive strain such as EGY191, on the theory that it is most important to get stronger interactors first and a complete profile of interactors later. Finally, some investigators prefer to work up the entire set of positives initially obtained, even if such positives number in the hundreds. Particularly in this latter case, it is most effective to use Alternate Protocol 1 as a means to identify unique versus common positives.

Isolate plasmid from positive colonies by transfer into E. coli

20a. Transfer yeast plasmids directly into *E. coli* by following the protocol for direct electroporation (*UNIT 1.8*, Alternate Protocol 2). Proceed to step 22.

20b. Isolate plasmid DNA from yeast by the rapid miniprep protocol (*UNIT 13.11*) with the following alteration: after obtaining aqueous phase, precipitate by adding sodium acetate to 0.3 M final and 2 vol ethanol, incubate 20 min on ice, microcentrifuge 15 min at maximum speed, wash pellet with 70% ethanol, dry, and resuspend in 5 µl TE buffer.

Cultures can be grown prior to the miniprep using Glu/CM −Trp to select only for the library plasmid; this may increase the proportion of bacterial colonies that contain the desired plasmid.

21. Use 1 µl DNA to electroporate (*UNIT 1.8*) into competent KC8 bacteria, and plate on LB/ampicillin plates. Incubate overnight at 37°C.

Electroporation must be used to obtain transformants with KC8 because the strain is generally refractory to transformation.

22. Restreak or replica plate colonies arising on LB/ampicillin plates to bacterial defined minimal A medium plates containing vitamin B1 and supplemented with Ura, His, and Leu but lacking Trp. Incubate overnight at 37°C.

Colonies that grow under these conditions contain the library plasmid.

The yeast TRP1 gene can successfully complement the bacterial trpC-9830 mutation, allowing the library plasmid to be easily distinguished from the other two plasmids contained in the yeast. It is helpful to first plate transformations on LB/ampicillin plates, which provides a less stringent selection, followed by restreaking to bacterial minimal medium to maximize the number of colonies obtained (E.G., unpub. observ.).

23. Purify library-containing plasmids using a bacterial miniprep procedure (*UNIT 1.6*).

Some investigators are tempted to immediately sequence DNAs obtained at this stage. At this point, it is still possible that none of the isolated clones will express bona fide interactors, and it is suggested that the following specificity tests be completed before committing the effort to sequencing (also see annotation to step 28).

Because multiple 2μm plasmids with the same marker can be simultaneously tolerated in yeast, it sometimes happens that a single yeast will contain two or more different library plasmids, only one of which encodes an interacting protein. The frequency of this occurrence varies in the hands of different investigators and may in some cases account for disappearing positives if the wrong cDNA is picked. When choosing colonies to miniprep, it is generally useful to work up at least two individual bacterial transformants for each yeast positive. These minipreps can then be restriction digested (UNIT 3.1) with EcoRI + XhoI to release cDNA inserts, and the size of inserts determined on an agarose minigel (UNIT 2.5A) to confirm that both plasmids contain the same insert. An additional benefit of analyzing insert size is that it may provide some indication as to whether repeated isolation of the same cDNA is occurring, generally a good indication concerning the biological relevance of the interactor. See Background Information for further discussion.

Assess positive colonies with specificity tests

Much spurious background will have been removed by the previous series of controls. Other classes of false positives can be eliminated by retransforming purified plasmids into "virgin" *LexA*-operator-*LEU2*/*LexA*-operator-*lacZ*/pBait–containing strains that have not been subjected to Leu selection and verifying that interaction-dependent phenotypes are still observed. Such false positives could include mutations in the initial EGY48 yeast that favor growth on Gal medium, library-encoded cDNAs that interact with the LexA DNA-binding domain, or proteins that are sticky and interact with multiple biologically unrelated fusion domains.

24. In separate transformations, use purified plasmids from step 23 to transform yeast that already contain the following plasmids and are growing on Glu/CM –Ura, –His plates:

    EGY48 containing pSH18-34 and pBait
    EGY48 containing pSH18-34 and pRFHM-1
    EGY48 containing pSH18-34 and a nonspecific bait (optional).

25. Plate each transformation mix on Glu/CM –Ura, –His, –Trp dropout plates and incubate 2 to 3 days at 30°C until colonies appear.

26. Create a Glu/CM –Ura, –His, –Trp master dropout plate for each library plasmid being tested. Streak adjacently five or six independent colonies derived from each of the transformation plates. Incubate overnight at 30°C.

27. Restreak or replica plate from this master dropout plate to the same series of test plates used for the actual screen:

    Glu/Xgal/CM –Ura, –His, –Trp
    Gal/Raff/Xgal/CM –Ura, –His, –Trp
    Glu/CM –Ura, –His, –Trp, –Leu
    Gal/CM –Ura, –His, –Trp, –Leu.

    True positive cDNAs should make cells blue on Gal/Raff/Xgal but not on Glu/Xgal plates, and should make them grow on Gal/Raff/CM –Leu but not Glu/CM –Leu dropout plates only if the cells contain LexA-bait. cDNAs that meet such criteria are ready to be sequenced (see legend to Fig. 20.1.3 for primer sequence) or otherwise characterized. Those cDNAs that also encode proteins that interact with either RFHM-1 or another nonspecific bait should be discarded.

    It may be helpful to cross-check the isolated cDNAs with a database of cDNAs thought to be false positives. This database is available on the World Wide Web as a work in progress at http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork.html. cDNAs reported to this database are generally those isolated only once in a screen in which obviously true interactive partners were isolated multiple times, cDNAs that may interact with more than one bait, or cDNAs for which the interaction does not appear to make biological sense

in the context of the starting bait. Although some proteins in this database may ultimately turn out in fact to associate with the bait that isolated them, they are by default unlikely to possess a unique and interesting function in the context of that bait if they are well represented in the database.

28. If appropriate, conduct additional specificity tests (see Support Protocol 5). Analyze and sequence positive isolates.

    The primer sequence for use with pJG4-5 is provided in the legend to Figure 20.1.4.

    DNA prepared from KC8 is generally unsuitable for dideoxy or automated sequencing even after use of Qiagen columns and/or cesium chloride gradients. Library plasmids to be sequenced should be retransformed from the KC8 miniprep stock (step 23) to a more amenable strain, such as DH5α, before sequencing is attempted.

RAPID SCREEN FOR INTERACTION TRAP POSITIVES

ALTERNATE PROTOCOL 1

Under some circumstances, it may be desirable to attempt the analysis of a large number of positives resulting from a two-hybrid screen. One such hypothetical example would be a bait with a leucine zipper or coiled coil known to dimerize with partner "A" that is highly expressed. In order to identify the rare novel partner "B", it is necessary to work through the high background of "A" reisolates. This protocol uses the polymerase chain reaction (PCR) in a strategy to sort positives into redundant (multiple isolates) and unique classes prior to plasmid rescue from yeast, thus greatly reducing the number of plasmid isolations that must be performed. An additional benefit is that this protocol preidentifies positive clones containing one or multiple library plasmids; for those containing only one library plasmid, only a single colony needs to be prepared through KC8/DH5α.

Additional Materials *(also see Basic Protocol 2)*

Yeast plated on Glu/CM –Ura, –His, –Trp master plate (see Basic Protocol 2, step 19)
Lysis solution (see recipe)
10 µM forward primer (FP1): 5′-CGT AGT GGA GAT GCC TCC-3′
10 µM reverse primer (FP2): 5′-CTG GCA AGG TAG ACA AGC CG-3′

Toothpicks or bacterial inoculating loops (*UNIT 1.1*), sterile
96-well microtiter plate
Sealing tape, e.g., wide transparent tape
150- to 212-µm glass beads, acid-washed (*UNIT 13.13*)
Vortexer with flat plate

Additional reagents and equipment for performing an interactor hunt (see Basic Protocol 2), PCR amplification of DNA (*UNIT 15.1*), agarose gel electrophoresis (*UNITS 2.5A & 2.6*), restriction endonuclease digestion (*UNIT 3.1*), electroporation (*UNIT 1.8*), and miniprep isolation of bacterial DNA (*UNIT 1.6*)

1. Perform an interactor hunt (see Basic Protocol 2, steps 1 to 19).

2. Use a sterile toothpick or bacterial inoculating loop to transfer yeast from the Glu/CM, –Ura, –His, –Trp master plate into 25 µl lysis solution in a 96-well microtiter plate. Seal the wells of the microtiter plate with sealing tape and incubate 1.5 to 3.5 hr at 37°C with shaking.

    The volume of yeast transferred should not exceed ~2 to 3 µl of packed pellet; larger quantities of yeast will reduce quality of the DNA. DNA can be efficiently recovered from master plates that have been stored up to 1 week at 4°C. If yeast have been previously gridded on master plates, transfer to microtiter plates can be facilitated by using a multicolony replicator.

3. Remove tape from the plate, add ~25 µl acid-washed glass beads to each well, and reseal with the same tape. Firmly attach the microtiter plate to a flat-top vortexer, and vortex 5 min at medium-high power.

   The microtiter plate can be attached to the vortexer using 0.25-in (0.64-cm) rubber bands.

4. Remove the tape and add ~100 µl sterile water to each well. Swirl gently to mix, then remove sample for step 5. Press the tape back firmly to seal the microtiter plate and place in the freezer at –20°C for storage.

5. Amplify 0.8 to 2.0 µl of sample by standard PCR (*UNIT 15.1*) in a ~30-µl volume using 3 µl each of the forward primer FP1 and the reverse primer FP2. Perform PCR using the following cycles:

Initial step:	2 min	94°C
31 cycles:	45 sec	94°C
	45 sec	56°C
	45 sec	72°C.

   These conditions have been used successfully to amplify fragments up to 1.8 kb in length; some modifications, such as extension of elongation time, are also effective.

6. Load 20 µl of the PCR reaction product on a 0.7% low melting temperature agarose gel (*UNIT 2.6*) to resolve PCR products. Based on insert sizes, group the obtained interactors in families, i.e., potential multiple independent isolates of identical cDNAs. Reserve gel until results of step 7 are obtained.

   No special precautions are needed for storing the gel. Since HaeIII digests typically yield rather small DNA fragments, running the second gel does not take a lot of time. Usually, the delay does not exceed 45 to 60 min, during which time the first gel may be stored in a gel box at room temperature or wrapped in plastic wrap at 4°C.

7. While the gel is running, use the remaining 10 µl of PCR reaction product for a restriction endonuclease digestion with *Hae*III in a digestion volume of ~20 µl (*UNIT 3.1*). Based on analysis of the sizes of undigested PCR products in the gel (step 6), rearrange the tubes with *Hae*III digest samples so that those thought to represent a family are side by side. Resolve the digests on a 2% to 2.5% agarose gel (*UNIT 2.5A*).

   Most restriction fragments will be in the 200-bp to 1.0-kb size range so using a long gel run is advisable. This analysis should produce a distinct fingerprint of insert sizes and allow definition of library cDNAs as unique isolates or related groups.

   A single positive yeast will sometimes contain multiple library plasmids. An advantage of this protocol is the ready detection of multiple library plasmids in PCR reactions; thus, following subsequent bacterial transformations, only a single TRP1 colony would need to be analyzed unless multiple plasmids were already known to be present.

8. Isolate DNA fragments from the low melting temperature agarose gel (step 6).

   If inspection of the banding pattern on the two gels suggests that a great many reisolates of a small number of cDNAs are present, it may be worthwhile to immediately sequence PCR products representative of these clusters, but it is generally still advisable to continue through specificity tests before doing so. If the PCR products are sequenced, the FP1 forward primer works well in automated sequencing of PCR fragments, but the FP2 primer is only effective in sequencing from purified plasmid.

   In general, priming from the AT-rich ADH terminator downstream of the polylinker/cDNA in library plasmid is less efficient than from upstream of the cDNA, and it is hard to design effective primers in this region.

9. Remove the microtiter plate of lysates from the freezer, thaw it, and remove 2 to 4 µl of lysed yeast for each desired positive. Electroporate DNA into either DH5α or KC8 *E. coli* as appropriate, depending on the choice of bait and reporter plasmids (see

Table 20.1.1 and see Background Information for further information). Refreeze the plate as a DNA reserve in case bacteria fail to transform on the first pass.

KC8 E. coli should be used for electroporation when the original reagents pEG202/pJG4-5/pJK101 are used for the interaction trap.

An additional strength of this protocol is that it identifies redundant clones before transfer of plasmids to bacteria, thus reducing the amount of work required in cases where plasmid identity can be unambiguously assigned. However, although restriction endonuclease digestion and PCR analysis are generally highly predictive, they are not 100% certain methods for estimating cDNA identity. Thus, if there is any doubt about whether two cDNAs are the same, investigators are urged to err on the side of caution.

10. Prepare a miniprep of plasmid DNA from the transformed bacteria (*UNIT 1.6*) and perform yeast transformation and specificity assessment (see Basic Protocol 2, steps 24 to 28).

PERFORMING A HUNT BY INTERACTION MATING

ALTERNATE PROTOCOL 2

An alternative way of conducting an interactor hunt is to mate a strain that expresses the bait protein with a strain that has been pretransformed with the library DNA, and screen the resulting diploid cells for interactors (Bendixen et al., 1994; Finley and Brent, 1994). This "interaction mating" approach can be used for any interactor hunt, and is particularly useful in three special cases. The first case is when more than one bait will be used to screen a single library. Interaction mating allows several interactor hunts with different baits to be conducted using a single high-efficiency yeast transformation with library DNA. This can be a considerable savings, since the library transformation is one of the most challenging tasks in an interactor hunt. The second case is when a constitutively expressed bait interferes with yeast viability. For such baits, performing a hunt by interaction mating avoids the difficulty associated with achieving a high-efficiency library transformation of a strain expressing a toxic bait. Moreover, the actual selection for interactors will be conducted in diploid yeast, which are more vigorous than haploid yeast and can better tolerate expression of toxic proteins. The third case is when a bait cannot be used in a traditional interactor hunt using haploid yeast strains (see Basic Protocol 2) because it activates transcription of even the least sensitive reporters. In diploids the reporters are less sensitive to transcription activation than they are in haploids. Thus, the interaction mating hunt provides an additional method to reduce background from transactivating baits.

In the protocol described below, the library DNA is used to transform a strain with a *LEU2* reporter (e.g., EGY48). This pretransformed library strain is then frozen in many aliquots, which can be thawed and used for individual interactor hunts. The bait is expressed in a strain of mating type opposite to that of the pretransformed library strain, and also bearing the *lacZ* reporter. A hunt is conducted by mixing the pretransformed library strain with the bait strain and allowing diploids to form on YPD medium overnight. The diploids are then induced for expression of the library-encoded proteins and screened for interactors as in Basic Protocol 2.

NOTE: Strain combinations other than those described below can also be used in an interaction-mating hunt. The key to choosing the strains is to ensure that the bait and prey strains are of opposite mating types and that both have auxotrophies to allow selection for the appropriate plasmids and reporter genes. Also, once the bait plasmid and *lacZ* reporter plasmid have been introduced into the bait strain, and the library plasmids have been introduced into the library strain, the resulting bait strain and library strain must each have auxotrophies that can be complemented by the other, so that diploids can be selected.

Additional Materials *(also see Basic Protocols 1 and 2)*

Yeast strains: either RFY206 (Finley and Brent, 1994), YPH499 (Sikorski and Hieter, 1989; ATCC #6625), or an equivalent *MATa* strain with auxotrophic markers *ura3*, *trp1*, *his3*, and *leu2*

YPD liquid medium (*UNIT 13.1*)

Glu/CM –Trp plates: CM dropout plates –Trp (*UNIT 13.1*) supplemented with 2% glucose

pJG4-5 library vector (Fig. 20.1.6), empty

100-mm YPD plates (*UNIT 13.1*)

Additional reagents and equipment for lithium acetate transformation of yeast (*UNIT 13.7*)

Construct the bait strain

The bait strain will be a *MATa* yeast strain (mating type opposite of EGY48) containing a *lacZ* reporter plasmid like pSH18-34 and the bait-expressing plasmid, pBait.

1. Perform construction of the bait plasmid (pBait; see Basic Protocol 1, step 1).

2. Cotransform the *MATa* yeast strain (e.g., either RFY206 or YPH499) with pBait and pSH18-34 using the lithium acetate method (*UNIT 13.7*). Select transformants on Glu/CM –Ura,–His plates by incubating plates at 30°C for 3 to 4 days until colonies form. Combine 3 colonies for all future tests and for the mating hunt.

   The bait strain (RFY206/pSH18-34/pBait or YPH499/pSH18-34/pBait) can be tested by immunoblotting to ensure that the bait protein is expressed (see Support Protocol 1). Synthesis and nuclear localization of the bait protein can also be tested by the repression assay (see Basic Protocol 1, steps 8 to 12).

3. *Optional:* Assay *lacZ* gene activation in the bait strain (see Basic Protocol 1, steps 4 to 7).

   If the bait activates the lacZ reporter, a less sensitive lacZ reporter plasmid (Table 20.1.1), or an integrated version of the lacZ reporter should be tried. A bait that strongly activates the lacZ reporters usually cannot be used in a hunt based on selection of interactors with the LEU2 reporter, because the LEU2 reporters are more sensitive than the lacZ reporters. However, both reporters are less sensitive to activation by a bait in diploid cells, as compared to haploid cells. Thus, a more important test of the transactivation potential of a bait is to test the leucine requirement of diploid cells expressing it, as described in steps 6 to 20, below.

Prepare the pretransformed library strain (EGY48 + library plasmids)

4. Perform a large-scale transformation of EGY48 with library DNA using the lithium acetate method (see Basic Protocol 2, steps 1 to 8, except start with EGY48 bearing no other plasmids). To prepare for transformation, grow EGY48 in YPD liquid medium. Select library transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

5. Collect primary transformants by scraping plates, washing yeast, and resuspending in 1 pellet vol glycerol solution (see Basic Protocol 2, steps 9 to 12). Freeze 0.2 to 1.0 ml aliquots at –70° to –80°C.

   The cells will be stable for at least 1 year. Refreezing a thawed aliquot will result in loss of viability. Thus, many frozen aliquots should be made, so that each thawed aliquot can be discarded after use.

Prepare the pretransformed control strain (EGY48 + pJG4-5)

6. Transform EGY48 grown in YPD liquid medium with the empty library vector, pJG4-5, using the lithium acetate method (*UNIT 13.7*). Select transformants on Glu/CM –Trp plates by incubating 3 days at 30°C.

7. Pick and combine three transformant colonies and use them to inoculate 30 ml of Glu/CM –Trp medium. Incubate 15 to 24 hr at 30°C (to OD_{600} >3).

8. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 10 ml sterile water to wash cells.

9. Centrifuge 5 min at 1000 to 1500 × *g*, room temperature, and remove supernatant. Resuspend in 1 pellet vol glycerol solution and freeze 100-µl aliquots at –70° to –80°C.

Determine plating efficiency of pretransformed library and pretransformed control strains

10. After freezing (at least 1 hr) thaw an aliquot of each pretransformed strain (from step 5 and step 9) at room temperature. Make several serial dilutions in sterile water, including aliquots diluted 10^5-fold, 10^6-fold, and 10^7-fold. Plate 100 µl of each dilution on 100-mm Glu/CM –Trp plates and incubate 2 to 3 days at 30°C.

11. Count the colonies and determine the number of colony-forming units (cfu) per aliquot of transformed yeast.

    The plating efficiency for a typical library transformation and for the control strain will be ~1 × 10^8 cfu per 100 µl.

Mate the bait strain with the pretransformed library strain and the pretransformed control strain

In steps 12 through 20, an interactor hunt is conducted concurrently with testing *LEU2* reporter activation by the bait itself. For most baits, this approach will be the quickest way to isolate interactors. However, for some baits, such as those that have a high transactivation potential, or those that affect yeast mating or growth, steps 12 through 20 will serve as a pilot experiment to determine the optimal parameters for a subsequent hunt.

12. Grow a 30-ml culture of the bait strain in Glu/CM –Ura,–His liquid dropout medium to mid to late log phase (OD_{600} = 1.0 to 2.0, or 2 to 4 × 10^7cells/ml).

    A convenient way to grow the bait strain is to inoculate a 5-ml culture with approximately three colonies from a plate and grow it overnight at 30°C with shaking. In the morning, measure the OD_{600}, dilute into a 30-ml culture to a final OD_{600} = 0.2, and grow at 30°C with shaking. The culture should reach mid to late log phase before the end of the day.

13. Centrifuge the culture 5 min at 1000 to 1500 × *g*, room temperature, to harvest cells. Resuspend the cell pellet in sterile water to make a final volume of 1 ml.

    This should correspond to ~1 × 10^9 cells/ml.

14. Set up two matings. In one sterile microcentrifuge tube mix 200 µl of the bait strain with 200 µl of a thawed aliquot of the pretransformed control strain from step 9. In a second microcentrifuge tube mix 200 µl of the bait strain with ~1 × 10^8 cfu (~0.1 to 1 ml) of the pretransformed library strain from step 5.

    The library mating should be set up so that it contains a ~2-fold excess of bait strain cfu over pretransformed library strain cfu. Because the bait strain was harvested in log phase, most of the cells will be viable (i.e., cells/ml = ~cfu/ml), and the number of cfu can be sufficiently estimated from optical density (1 OD_{600} = ~2 × 10^7 cells/ml). Under these conditions, ~10% of the cfu in the pretransformed library strain will mate with the bait

strain. Thus, a complete screen of 10^7 library transformants will require a single mating with at least 10^8 cfu of the pretransformed library strain and at least 2×10^8 cfu of the bait strain.

To screen more library transformants, set up additional matings. The number of pretransformed library transformants to screen depends on the size of the library and the number of primary transformants obtained in step 5. If the size of the library is larger than the number of transformants obtained in step 5, the goal will be to screen all of the yeast transformants. In this case, complete screening of the library will require additional transformations of EGY48 and additional interactor hunts. If the size of the library is smaller than the number of transformants obtained in step 5, the goal will be to screen at least a number of transformants equivalent to the size of the library.

15. Centrifuge each cell mixture for 5 min at 1000 to 1500 × *g*, pour off medium, and resuspend cells in 200 µl YPD medium. Plate each suspension on a 100-mm YPD plate. Incubate 12 to 15 hr at 30°C.

16. Add ~1 ml of Gal/Raff/CM –Ura, –His, –Trp to the lawns of mated yeast on each plate. Mix the cells into the medium using a sterile applicator stick.

17. Transfer each slurry of mated cells to a 500-ml flask containing 100 ml of Gal/Raff/CM –Ura, –His, –Trp dropout medium. Incubate with shaking 6 hr at room temperature to induce the *GAL1* promoter, which drives expression of the cDNA library.

18. Centrifuge the cell suspensions 5 min at 1000 to 1500 × *g*, room temperature, to harvest the cells. Wash by resuspending in 30 ml of sterile water and centrifuging again. Resuspend each pellet in 5 ml sterile water. Measure OD_{600} and, if necessary, dilute to a final concentration of $\sim 1 \times 10^8$ cells/ml.

    This is a mixture consisting of haploid cells that have not mated and diploid cells. Under a microscope, the two cell types can be distinguished by size (diploids are ~1.7× bigger than haploids) and shape (diploids are slightly oblong and haploids are spherical). Because diploids grow faster than haploids, this mixture will contain ~10% to 50% diploid cells. The actual number of diploids will be determined by plating dilutions on –Ura, –His, –Trp medium, which will not support the growth of the parental haploids.

19. For each mating make a series of $1/10$ dilutions in sterile water, at least 200 µl each, to cover a 10^6-fold concentration range. Plate 100 µl from each tube (undiluted, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} dilution) on 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Plate 100 µl from the 10^{-4}, 10^{-5}, and 10^{-6} tubes on 100-mm Gal/Raff/CM –Ura, –His, –Trp plates. Incubate plates at 30°C. Count the colonies on each plate after 2 to 5 days.

20. For the mating with the pretransformed library, prepare an additional 3 ml of a 10^{-1} dilution. Plate 100 µl of the 10^{-1} dilution on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Also plate 100 µl of the undiluted cells on each of 20 100-mm Gal/Raff/CM –Ura, –His, –Trp, –Leu plates. Incubate at 30°C. Pick Leu^+ colonies after 2 to 5 days and characterize them beginning with step 17 of Basic Protocol 2.

    The number of Leu^+ colonies to pick to ensure that all of the pretransformed library has been screened depends on the transactivation potential of the bait protein itself. The transactivation potential is expressed as the number of Leu^+ colonies that grow per cfu (Leu^+/cfu) of the bait strain mated with the control strain, as determined in step 19 of this protocol. It can be calculated as the ratio of the number of colonies that grow on Gal/Raff/CM –Ura,–His, –Trp, –Leu to the number of colonies that grow on Gal/Raff/CM –Ura, –His, –Trp for a given dilution of the mating between the bait strain and the control strain. A bait with essentially no transactivation potential will produce less than 10^{-6} Leu^+/cfu. For a bait to be useful in an interactor hunt it should not transactivate more than 10^{-4} Leu^+/cfu.

To screen all of the pretransformed library, it will be necessary to pick a sufficient number of Leu^+ colonies in addition to background colonies produced by the transactivation potential of the bait itself. Thus, the minimum number of Leu^+ colonies that should be picked in step 20 of this protocol is given by:

(transactivation potential, Leu^+/cfu) × (# library transformants screened).

For example, if 10^7 library transformants were obtained in step 2 (and at least 10^8 cfu of these transformants were mated with the bait strain in step 14, since only ~10% will form diploids), and the transactivation potential of the bait is 10^{-4} Leu^+/cfu, then at least 1000 Leu^+ colonies must be picked and characterized. In other words, if the rarest interactor is present in the pretransformed library at a frequency of 10^{-7}, to find it one needs to screen through at least 10^7 diploids from a mating of the library strain. However, at least 1000 of these 10^7 diploids would be expected to be Leu^+ due to the bait background if the transactivation potential of the bait is 10^{-4}. The true positives will be distinguished from the bait background in the next step by the galactose dependence of their Leu^+ and $lacZ^+$ phenotypes.

PREPARATION OF PROTEIN EXTRACTS FOR IMMUNOBLOT ANALYSIS

SUPPORT PROTOCOL 1

To confirm that the bait fusion protein constructed in Basic Protocol 1 is synthesized properly, a crude lysate is prepared for SDS-PAGE and immunoblot analysis (*UNITS 10.2 & 10.8*). The presence of the target protein is detected by antibody to LexA or the fusion domain.

Materials

Master plates with pBait-containing positive and control yeast on Glu/CM –Ura, –His dropout medium (see Basic Protocol 1, step 4)

Glu/CM –Ura, –His dropout liquid medium: CM dropout plates –Ura, –His (*UNIT 13.1*) supplemented with 2% glucose

2× Laemmli sample buffer (see recipe)

Antibody to fusion domain or LexA: monoclonal antibody to LexA (Clontech, Invitrogen) or polyclonal antibody to LexA (available by request from R. Brent or E. Golemis)

30°C incubator

100°C water bath

Additional reagents and equipment for SDS-PAGE (*UNIT 10.2*) and immunoblotting and immunodetection (*UNIT 10.8*)

1. From the master plates, start a 5-ml culture in Glu/CM –Ura, –His liquid medium for each bait being tested and for a positive control for protein expression (i.e., RFHMI or SH17-4). Incubate overnight at 30°C.

   For each construct assayed, it is a good idea to grow colonies from at least two primary transformants, as levels of bait expression are sometimes heterogenous.

2. From each overnight culture, start a fresh 5-ml culture in Glu/CM –Ura, –His at OD_{600} = ~0.15. Incubate again at 30°C.

3. When the culture has reached OD_{600} = 0.45 to 0.7 (~4 to 6 hr), remove 1.5 ml to a microcentrifuge tube.

   For some LexA fusion proteins, levels of the protein drop off rapidly in cultures approaching stationary phase. This is due to a combination of the diminishing activity of the ADH1 promoter in late growth phases and the relative instability of particular fusion domains. Thus, it is not a good idea to let cultures become saturated in the hopes of obtaining a higher yield of protein.

4. Microcentrifuge cells 3 min at 13,000 × *g*, room temperature. When the pellet is visible, remove the supernatant.

   Inspection of the tube should reveal a pellet ~1 to 3 µl in volume. If the pellet is not visible, microcentrifuge another 3 min.

5. Working rapidly, add 50 µl of 2× Laemmli sample buffer to the visible pellet in the tube, vortex, and place the tube on dry ice.

   Samples may be frozen at –70°C.

6. Transfer frozen sample directly to a boiling water bath or a PCR machine set to cycle at 100°C. Boil 5 min.

7. Microcentrifuge 5 sec at maximum speed to pellet large cellular debris.

8. Perform SDS-PAGE (*UNIT 10.2*) using 20 to 50 µl sample per lane.

9. To detect the protein, immunoblot and analyze (*UNIT 10.8*) using antibody to the fusion domain or LexA.

SUPPORT PROTOCOL 2

PREPARATION OF SHEARED SALMON SPERM CARRIER DNA

This protocol generates high-quality sheared salmon sperm DNA (sssDNA) for use as carrier in transformation (Basic Protocol 2). This DNA is also suitable for other applications where high-quality carrier DNA is needed (e.g., hybridization). This protocol is based on Schiestl and Gietz (1989). For more details of phenol extraction or other DNA purification methods, consult *UNIT 2.1A*.

Materials

High-quality salmon sperm DNA (e.g., sodium salt from salmon testes, Sigma or Boehringer Mannheim), desiccated
TE buffer, pH 7.5 (*APPENDIX 2*), sterile
TE-saturated buffered phenol (*UNIT 2.1A*)
1:1 (v/v) buffered phenol/chloroform
Chloroform
3 M sodium acetate, pH 5.2 (*APPENDIX 2*)
100% and 70% ethanol, ice cold

Magnetic stirring apparatus and stir-bar, 4°C
Sonicator with probe
50-ml conical centrifuge tube
High-speed centrifuge and appropriate tube
100°C and ice-water baths

1. Dissolve desiccated high-quality salmon sperm DNA in TE buffer, pH 7.5, at a concentration of 5 to 10 mg/ml by pipetting up and down in a 10-ml glass pipet. Place in a beaker with a stir-bar and stir overnight at 4°C to obtain a homogenous viscous solution.

   It is important to use high-quality salmon sperm DNA. Sigma Type III sodium salt from salmon testes has worked well, as has a comparable grade from Boehringer Mannheim. Generally it is convenient to prepare 20- to 40-ml batches at a time.

2. Shear the DNA by sonicating briefly using a large probe inserted into the beaker.

   The goal of this step is to generate sheared salmon sperm DNA (sssDNA) with an average size of 7 kb, but ranging from 2 to 15 kb. Oversonication (such that the average size is closer to 2 kb) drastically decreases the efficacy of carrier in enhancing transformation. The original version of this protocol (Schiestl and Gietz, 1989) called for two 30-sec pulses at

Table 20.1.4 Two-hybrid System Variants[a]

System	DNA-binding domain	Activation domain	Selection	Reference
Two-hybrid	*GAL4*	*GAL4*	Activation of *lacZ*, *HIS3*	Chien et al., 1991
Interaction trap	*LexA*	*B42*	Activation of *LEU2*, *lacZ*	Gyuris et al., 1993
"Improved two-hybrid"	*GAL4*	*GAL4*	Activation of *HIS3*, *lacZ*	Durfee at al., 1993
Modified two-hybrid	*LexA*	VP16	Activation of *HIS3*, *lacZ*	Vojtek at al., 1993
KISS	*GAL4*	VP16	Activation of *CAT*, *hyg*^{r}	Fearon et al., 1992
Contingent replication	*GAL4*	VP16	Activation of T-Ag, replication of plasmids	Vasavada et al., 1991

[a]Abbreviations: *CAT*, chloramphenicol transferase gene; *hyg*^{r}, hygromycin resistance gene; T-Ag, viral large T antigen.

three-quarter power, but optimal conditions vary between sonicators. The first time this protocol is performed, it is worthwhile to sonicate briefly, then test the size of the DNA by running out a small aliquot alongside molecular weight markers on an agarose gel containing ethidium bromide. The DNA can be sonicated further if needed.

3. Once DNA of the appropriate size range has been obtained, extract the sssDNA solution with an equal volume of TE-saturated buffered phenol in a 50-ml conical tube, shaking vigorously to mix.
4. Centrifuge 5 to 10 min at 3000 × *g*, room temperature, or until clear separation of phases is obtained. Transfer the upper phase containing the DNA to a clean tube.
5. Repeat extraction using 1:1 (v/v) buffered phenol/chloroform, then chloroform alone. Transfer the DNA into a tube suitable for high-speed centrifugation.
6. Precipitate the DNA by adding $\frac{1}{10}$ vol of 3 M sodium acetate and 2.5 vol of ice-cold 100% ethanol. Mix by inversion. Centrifuge 15 min at ~12,000 × *g*, room temperature.
7. Wash the pellet with 70% ethanol. Briefly dry either by air drying, or by covering one end of the tube with Parafilm with a few holes poked in and placing the tube under vacuum. Resuspend the DNA in sterile TE buffer at 5 to 10 mg/ml.

   Do not overdry the pellet or it will be very difficult to resuspend.
8. Denature the DNA by boiling 20 min in a 100°C water bath. Then immediately transfer the tube to an ice-water bath.
9. Place aliquots of the DNA in microcentrifuge tubes and store frozen at –20°C. Thaw as needed.

   DNA should be boiled again briefly (5 min) immediately before addition to transformations.

   Before using a new batch of sssDNA in a large-scale library transformation, it is a good idea to perform a small-scale transformation using suitable plasmids to determine the transformation efficiency. Optimally, use of sssDNA prepared in the manner described will yield transformation frequencies of >10^5 colonies/μg input plasmid DNA.

SUPPORT PROTOCOL 3

YEAST COLONY HYBRIDIZATION

This protocol is adapted from a modification of the classic protocol of Grunstein and Hogness (1975; Kaiser et al., 1994). It is primarily useful when a large number of putative interactors has been obtained, and initial minipreps and restriction digests have indicated that many of them derive from a small number of cDNAs; these cDNAs can then be used as probes to screen and eliminate identical cDNAs from the pool.

Materials

Glu/CM –Trp plates: CM dropout plates –Trp (*UNIT 13.1*) supplemental with 2% glucose
Master dropout plate of yeast positive for Gal dependence (see Basic Protocol 2, step 18)
1 M sorbitol/20 mM EDTA/50 mM DTT (prepare fresh)
1 M sorbitol/20 mM EDTA
0.5 M NaOH
0.5 M Tris·Cl (pH 7.5)/6× SSC (*APPENDIX 2*)
2× SSC (*APPENDIX 2*)
100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma)

82-mm circular nylon membrane, sterile
Whatman 3MM paper
80°C vacuum oven *or* UV cross-linker

Additional reagents and equipment for bacterial filter hybridization (*UNITS 6.3 & 6.4*)

1. Place a sterile nylon membrane onto a Glu/CM –Trp dropout plate. From the master dropout plate of Gal-dependent positives, gently restreak positives to be screened onto the membrane and mark the membrane to facilitate future identification of hybridizing colonies. Grow overnight (~12 hr) at 30°C.

   Growth for extended periods of time (i.e., 24 hr) may result in difficulty in obtaining good lysis. It is a good idea to streak positive and negative controls for the cDNAs to be hybridized on the membrane.

2. Remove membrane from plate. Air dry briefly. Incubate ~30 min on a sheet of Whatman 3MM paper saturated with 1 M sorbitol/20 mM EDTA/50 mM DTT.

   Optionally, before commencing chemical lysis, membranes can be placed at –70°C for 5 min, then thawed at room temperature for one or more cycles to enhance cell wall breakage.

3. Cut a piece of Whatman 3MM paper to fit inside a 100-mm petri dish. Place the paper disc in the dish and saturate with 100,000 U/ml β-glucuronidase diluted 1:500 in 1 M sorbitol/20 mM EDTA (2 µl glucuronidase per ml of sorbitol/EDTA to give 200 U/ml final). Layer nylon membrane on dish, cover dish, and incubate up to 6 hr at 37°C until >80% of the cells lack a cell wall.

   The extent of cell wall removal can be determined by removing a small quantity of cells from the filter to a drop of 1 M sorbitol/20 mM EDTA on a microscope slide and observing directly with a phase-contrast microscope at ≥60× magnification. Cells lacking cell wall are nonrefractile.

4. Place membrane on Whatman 3MM paper saturated with 0.5 M NaOH for ~8 to 10 min.

5. Place membrane on Whatman 3MM paper saturated with 0.5 M Tris·Cl (pH 7.5)/6× SSC for 5 min. Repeat with a second sheet of Whatman 3MM paper.

6. Place membrane on Whatman 3MM paper saturated with 2× SSC for 5 min. Then place membrane on dry Whatman paper to air dry for 10 min.
7. Bake membrane 90 min at 80°C in vacuum oven or UV cross-link.
8. Process as for bacterial filter hybridization (*UNITS 6.3 & 6.4*), hybridizing the membrane with probes complementary to previously isolated cDNAs.

When selecting probes, either random-primed cDNAs or oligonucleotides complementary to the cDNA sequence may be used. If the cDNA is a member of a protein family, it may be advantageous to use oligonucleotides to avoid inadvertently excluding genes related but not identical to those initially obtained.

MICROPLATE PLASMID RESCUE

SUPPORT PROTOCOL 4

In some cases, it is desirable to isolate plasmids from a large number of positive colonies (Basic Protocol 2, steps 18 and 19). The protocol described below is a batch DNA preparation protocol developed by Steve Kron (University of Chicago, Chicago, Ill.) as a scale-up of a basic method developed by Manuel Claros (Laboratoire de Génétique Moleculaire, Paris, France).

Materials

2× Glu/CM –Trp liquid medium: 2× CM –Trp liquid medium (*UNIT 13.1*) supplemented with 4% glucose
Master plate of Gal-dependent yeast colonies (see Basic Protocol 2, step 18)
Rescue buffer: 50 mM Tris·Cl (pH 7.5)/10 mM EDTA/0.3% (v/v) 2-mercaptoethanol (prepare fresh)
Lysis solution: 2 to 5 mg/ml Zymolyase 100T/rescue buffer *or* 100,000 U/ml β-glucuronidase (type HP-2 crude solution from *Helix pomatia*; Sigma) diluted 1:50 in rescue buffer
10% (w/v) SDS
7.5 M ammonium acetate (*APPENDIX 2*)
Isopropanol
70% ethanol
TE buffer, pH 8.0 (*APPENDIX 2*)

24-well microtiter plates
Centrifuge with microplate holders, refrigerated
Repeating micropipettor
37°C rotary shaker

Grow yeast cultures

1. Aliquot 2 ml of 2× Glu/CM –Trp medium into each well of a 24-well microtiter plate. Into each well, pick a putative positive colony. Grow overnight with shaking at 30°C.

The 2× minimal medium is used to maximize the yield of yeast. Four plates can generally be handled conveniently at once, based on the number that can be centrifuged simultaneously.

2. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant with a snap and return the plate to upright.
3. Swirl or lightly vortex the plate to resuspend cell pellets in remaining liquid. Add 1 ml water to each well and swirl lightly.

Cell pellets can most easily be resuspended in residual liquid before adding new solutions. Addition of liquid can be accomplished using a repeating pipettor.

4. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet. Add 1 ml rescue buffer.
5. Centrifuge 5 min at 1500 × *g*, 4°C. Shake off supernatant and resuspend pellet in the small volume of liquid remaining in the plate.

Lyse cells

6. To each well, add 25 µl lysis solution. Swirl or vortex to mix. Incubate (with cover on) on a rotary shaker ~1 hr at 37°C.

   Lysis solution need not be completely dissolved before use. By 1 hr, lysis should be obvious as coagulation of yeast into a white precipitate.

   Susceptibility of yeast strains to lytic enzymes varies. If lysis occurs rapidly, then less lytic enzyme should be used. If the lysis step is allowed to go too far, too much of the partially dissolved cell wall may contaminate the final material. Lysis can be judged by examining cells with a phase-contrast microscope. Living cells are white with a dark halo and dead cells are uniformly gray. Lysis leads to release of granular cell contents into the medium. Once cells are mostly gray and many are disrupted, much of the plasmid should have been released.

7. To each well, add 25 µl of 10% SDS. Mix gently by swirling to completely disperse the precipitates. Allow plates to sit 1 min at room temperature.

   At this point, the wells should contain a clear, somewhat viscous solution.

Purify plasmid

8. To each well, add 100 µl of 7.5 M ammonium acetate. Swirl gently, then incubate 15 min at –70°C or –20°C until frozen.

   Addition of acetate should result in the formation of a massive white precipitate of cell debris and SDS. The freezing step appears to improve removal of inhibitors of E. coli transformation.

9. Remove plate from freezer. Once it begins to thaw, centrifuge 15 min at 3000 × *g*, 4°C. Transfer 100 to 150 µl of the resulting clear supernatants to clean 24-well plates.

   In general, some contamination of the supernatant with pelleted material cannot be avoided. However, it is better to sacrifice yield in order to maintain purity.

10. To each well, add ~0.7 vol isopropanol. Mix by swirling and allow to precipitate 2 min at room temperature.

    A cloudy fine precipitate should form immediately after isopropanol is added.

11. Centrifuge 15 min at 3000 × *g*, 4°C. Shake off supernatant with a snap.
12. To each well, add ~1 ml cold 70% ethanol. Mix by swirling, centrifuge 5 min at 3000 × *g*, 4°C. Shake off supernatant with a snap, invert plates and blot well onto paper towel. Allow plates to air dry.
13. To each well, add 100 µl TE buffer. Swirl well and allow to rest on bench several minutes, until the pellets appear fully dissolved. Transfer preps to microcentrifuge tubes or 96-well plates for storage at –20°C.

    One to five microliters of each of the resulting preparations can be used to transform competent E. coli: for KC8, electroporation should be used (see Basic Protocol 2, step 21). Sometimes, the yield of transformants is low if E. coli carrying plasmids are not permitted time to increase the plasmid copy number above a critical threshold before the cells are placed on selective medium. Allow plenty of time for cells to express antibiotic resistance or the TRP1 gene before plating.

    If insufficient numbers of colonies are obtained by this approach, the final plasmid preparation can be resuspended in 20 µl instead of 100 µl TE buffer to concentrate the DNA stock.

ADDITIONAL SPECIFICITY SCREENING

The three test plasmids outlined (pSH18-34, pRFHM1, and pEG202; see Basic Protocol 2, step 24) represent a minimal test series. If other LexA-bait proteins that are related to the bait protein used in the initial library screen are available, substantial amounts of information can be gathered by additional specificity tests. For example, if the initial bait protein was LexA fused to the leucine zipper of c-Fos, specificity screening of interactor-hunt positives against the leucine zippers of c-Jun or GCN4 in addition to that of c-Fos might allow discrimination between proteins that are specific for fos versus those that generically associate with leucine zippers.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see ***APPENDIX 2****; for suppliers, see* ***APPENDIX 4****.*

Glycerol solution

65% (v/v) glycerol, sterile
0.1 M $MgSO_4$
25 mM Tris·Cl, pH 8.0 (*APPENDIX 2*)
Store up to 1 year at room temperature

Laemmli sample buffer, 2×

10% (v/v) 2-mercaptoethanol (2-ME)
6% (w/v) SDS
20% (v/v) glycerol
0.2 mg/ml bromphenol blue
0.025× Laemmli stacking buffer (see recipe; optional)
Store up to 2 months at room temperature

This reagent can conveniently be prepared 10 ml at a time.

Laemmli stacking buffer, 2.5×

0.3 M Tris·Cl, pH 6.8
0.25% (w/v) SDS
Store up to 1 month at 4°C

Lysis solution

50 mM Tris·Cl, pH 7.5 (*APPENDIX 2*)
10 mM EDTA
0.3% (v/v) 2-mercaptoethanol (2-ME), added just before use
2% (v/v) β-glucuronidase from *Helix pomatia* (Type HP-2; Sigma), added just before use

COMMENTARY

Background Information

Interaction-based cloning is derived from three experimental observations. In the first, Brent and Ptashne (1985) demonstrated that it was possible to assemble a novel, functional transcriptional activator by fusing the DNA-binding domain from one protein, LexA, to the activation domain from a second protein, GAL4. This allowed the use of a single reporter system containing a single DNA-binding motif, the *LexA* operator, to study transcriptional activation by any protein of interest. In the second, Ma and Ptashne (1988) built on this work to demonstrate that the activation domain could be brought to DNA by interaction with a DNA-binding domain. In the third, Fields and Song (1989), working independently of Ma and Ptashne, used two yeast proteins, SNF1 and SNF4, to make an SNF1 fusion to the DNA-binding domain of GAL4 and an SNF4 fusion to the GAL4 activation domain. They demonstrated that the strength of the SNF1-SNF4

interaction was sufficient to allow activation through a GAL4 DNA-binding site. From this, they suggested the feasibility of selecting interacting proteins by performing screens of cDNA libraries made so that library-encoded proteins carried activating domains.

Several groups have developed cDNA library strategies along these lines, with some systems using LexA and others using GAL4 as the DNA-binding domain (Table 20.1.4). LexA and GAL4 each have different properties that should be considered when selecting a system. LexA is derived from a heterologous organism, has no known effect on the growth of yeast, possesses no residual transcriptional activity, can be used in GAL4$^+$ yeast, and can be used with a Gal-inducible promoter. Because GAL4 is an important yeast transcriptional activator, it has the disadvantage that experiments must be performed in gal4$^-$ yeast strains to avoid background due to activation of the reporter system by endogenous GAL4. Such gal4$^-$ strains are frequently less healthy and more difficult to transform than wild-type strains, and either libraries must be constitutively expressed or alternate inducible systems must be used. By contrast, the GAL4 DNA-binding domain may be more efficiently localized to the nucleus and may be preferred for some proteins (for a review of GAL4-based systems, see Bartel et al., 1993). Whichever system is used, it is important to remember that the bait protein constitutes a novel fusion protein whose properties may not exactly parallel those of the original unfused protein of interest. Although systems using the two-hybrid paradigm have been developed in mammalian cells (see Table 20.1.4), these have not been used effectively in library screens. It seems likely that the organism of choice for two-hybrid identification of novel partner proteins will remain yeast.

cDNAs that pass specificity tests are referred to as positives, or "true positives." In interactor hunts conducted to date, anywhere from zero to practically all isolated plasmids passed the final specificity test. If no positives are obtained, the tissue source for the library originally used may not be appropriate, and a different library may produce better results. However, there are some proteins for which no positives are found. Various explanations for this are provided below. Conversely, some library-encoded proteins are known to be isolated repeatedly using a series of unrelated baits, and these proteins demonstrate at least some specificity. One of these, heat shock protein 70, might be explained by positing that it assists the folding of some LexA-fused bait proteins, or alternatively, that these bait proteins are not normally folded. This example illustrates the point that the physiological relevance of even quite specific interactions may sometimes be obscure.

Because the screen involves plating multiple cells to Gal/CM –Ura, –His, –Trp, –Leu dropout medium for each primary transformant obtained, multiple reisolates of true positive cDNAs are frequently obtained. If a large number of specific positives are obtained, it is generally a good idea to attempt to sort them into classes—for example, digesting minipreps of positives with *Eco*RI, *Xho*I, and *Hae*III will generate a fingerprint of sufficient resolution to determine whether multiple reisolates of a small number of clones or single isolates of many different clones have been obtained. The former situation is a good indication that the system is working well.

An important issue that arises in an interactor hunt is the question of how biologically relevant interacting proteins that are isolated are likely to be. This leads directly to the question of what K_d of association two molecules must have to be detected by an interactor hunt. In fact, this is not at all a simple issue. For the system described here, most fusion proteins appear to be expressed at levels ranging from 50 nM to 1 μM (Golemis and Brent, 1992). Given the strength of the *GAL* promoter, it is likely that many library-encoded proteins are expressed at similarly high levels, ≥1 μM in the nucleus (Golemis and Brent, 1992). At this concentration, which is in considerable excess over the nuclear concentration of operator-bound bait protein, a cDNA library–encoded protein should half-maximally occupy the DNA-bound bait protein if it possesses a K_d of 10^{-6} M, making it theoretically possible that very-low-affinity interactions could be detected. Such interactions have been observed in some cases. In contrast, some interactions that have been previously established using other methods and are predicted by known K_d to be easily detected by these means, either are not detected or are detected only weakly (Finley and Brent, 1994; Estojak et al., 1995). Because of the conservation of many proteins between lower and higher eukaryotes, one explanation for this observation is that either one or both of the partners being tested is being sequestered from the desired interaction by fortuitous association with an endogenous yeast protein. A reasonably complete investigation of the degree of correlation between in vitro determina-

tions of interaction affinity and apparent strength of interaction in the interaction trap is included in Estojak et al. (1995). The result of this investigation suggests it is important to measure the affinity of detected interactions under different conditions, using a second assay system, rather than to draw conclusions about affinity based on detection in the interaction trap.

A number of different plasmids can be used for conducting an interactor hunt. Their properties are summarized in Tables 20.1.1 and 20.1.2. Because of the generous and open scientific exchange between investigators using the system, the number of available plasmids and other components has greatly expanded since the appearance of the initial two-hybrid reagents, facilitating the study of proteins inaccessible by the original system.

The original parent plasmid for generating LexA fusions, pEG202 is a derivative of 202 + PL (Ruden et al., 1991; see Fig. 20.1.3) that contains an expanded polylinker region. The available cloning sites in pEG202 include *Eco*RI, *Bam*HI, *Sal*I, *Nco*I, *Not*I, and *Xho*I, with the reading frame as described in the legend to Figure 20.1.3. Since the original presentation of this system, a number of groups have developed variants of this plasmid that address specialized research needs. Those currently available, as well as purposes for which they are suited, are listed in Table 20.1.1. pGilda, created by David A. Shaywitz, places the LexA-fusion cassette under the control of the inducible *GAL1* promoter, allowing expression of the bait protein for limited times during library screening, reducing the exposure of yeast to toxic baits. pJK202, created by Joanne Kamens, adds nuclear localization sequences to pEG202, facilitating assay of the function of proteins lacking internal nuclear localization sequences. pNLexA, created by Ian York, places LexA carboxy-terminal in the fusion domain, allowing assay of interactions that require an unblocked amino-terminus on the bait protein. pEE202I, created by Mike Watson and Rich Buckholz, allows chromosomal integration of a pEG202-like bait, thus reducing expression levels so they are more physiological for bait proteins normally present at low levels intracellularly. All of these have been extensively tested by numerous researchers. pGilda, pJK202, and pEE202I work with complete reliability. pNLexA works effectively with ~50% of the fusion domains tried, but synthesizes only very low levels of protein (relative to expression of the same fusion domain as a pEG202 fusion) with the remaining 50%. Attachment of fusion domains amino-terminal either to *LexA* or *GAL4* has been generally problematic in the hands of many investigators; it may be that appending additional protein sequences to the amino termini of these proteins is destabilizing, although the problem has not been rigorously investigated.

A series of *lacZ* reporters of differing sensitivity to transcriptional activation can be used to detect interactions of varying affinity (see Table 20.1.2). These plasmids are *LexA* operator–containing derivatives of the plasmid LR1Δ1 (West et al., 1984). In LR1Δ1, a minimal *GAL1* promoter lacking the *GAL1* upstream activating sequences (GAL_{UAS}) is located upstream of the bacterial *lacZ* gene. In pSH18-34, eight *LexA* operators have been cloned into an *Xho*I site located 167 bp upstream of the *lacZ* gene (S. Hanes, unpub. observ.). pJK103 and pRB1840 contain two and one operator, respectively.

pJK101 is similar to pSH18-34, except that it contains the *GAL1* upstream activating sequences (GAL_{UAS}) upstream of two *LexA* operator sites. A derivative of del20B (West et al., 1984), it is used in the repression assay (Brent and Ptashne, 1984; see Fig. 20.1.5) to assess LexA fusion binding to operator.

pSH17-4 is a *HIS3* 2μm plasmid that encodes LexA fused to the activation domain of the yeast activator GAL4. EGY48 cells bearing this plasmid will produce colonies in overnight growth on medium lacking Leu, and yeast that additionally contain pSH18-34 will turn deep blue on plates containing Xgal. This plasmid serves as a positive control for the activation of transcription.

pRFHM1 is a *HIS3* 2μm plasmid that encodes LexA fused to the N-terminus of the *Drosophila* protein bicoid. The plasmid has no ability to activate transcription, so EGY48 cells that contain pRFHM1 and pSH18-34 do not grow on –Leu medium and remain white on plates containing Xgal. pRFHM1 is a good control for specificity testing, because it has been demonstrated to be sticky—that is, to associate with a number of library-encoded proteins that are clearly nonphysiological interactors (R. Finley, Wayne State University, Detroit, Mich., unpub. observ.).

This protocol uses interaction libraries (Table 20.1.3) made in pJG4-5 or its derivatives (see Fig. 20.1.6). pJG4-5 was developed to facilitate isolation and characterization of novel proteins in interactor hunts (Gyuris et al., 1993). The pJG4-5 cDNA library expression

cassette is under control of the *GAL1* promoter, so library proteins are expressed in the presence of galactose (Gal) but not glucose (Glu). This conditional expression has a number of advantages, the most important of which is that many false-positives obtained in screens can be easily eliminated because they do not demonstrate a Gal-dependent phenotype. The expression cassette consists of an ATG to start translation, a nuclear localization signal to extend the interaction trap's range to include proteins that are normally predominantly localized in the cytoplasm, an activation domain (acid blob; Ma and Ptashne, 1987), the hemagglutinin epitope tag to permit rapid assessment of the size of encoded proteins, *Eco*RI-*Xho*I sites designed to receive directionally synthesized cDNAs, and the alcohol dehydrogenase (*ADH*) termination sequences to enhance the production of high levels of library protein. The plasmid also contains the *TRP1* auxotrophy marker and 2μm origin for propagation in yeast. A derivative plasmid, pJG4-5I, was created by Mike Watson and Richard Buckholz to facilitate chromosomal integration of the activation domain fusion expression plasmid.

A series of recently developed derivatives of pEG202, pJG4-5, and *lacZ* reporter plasmids (MW101 to MW112) alter the antibiotic resistance markers on these plasmids from ampicillin (Ap^r) to either kanamycin (Km^r) or chloramphenicol (Cm^r; Watson et al., 1996). Judiciously mixing and matching these plasmids in conjunction with Ap^r libraries would considerably reduce work subsequent to library screening, because the KC8 transformation, which involves *trpC* complementation in bacteria, could be omitted.

EGY48 and EGY191 (see Table 20.1.2) are both derivatives of the strain U457 (a gift of Rodney Rothstein, Columbia University, New York, N.Y.) in which the endogenous *LEU2* gene has been replaced by homologous recombination with *LEU2* reporters carrying varying numbers of *LexA* operators, using a procedure detailed in Estojak et al. (1995).

Interaction Trap–compatible reagents have recently become commercially available; Clontech and Invitrogen were the first to market such reagents and have recently been joined by OriGene. All suppliers use systems with the most sensitive reporters (EGY48 and pSH18-34), and provide their own positive and negative controls for testing activation or interaction between defined proteins. For expression of bait and library proteins, the Clontech Matchmaker LexA two-hybrid system and the OriGene Duplex-A system use some of the basic set of plasmids described here (see Table 20.1.1 for availability). Forward sequencing primers for bait and library plasmids are included in the Clontech kit, and Insert Screening Amplimer Sets for both plasmids can be acquired separately. Additional related products from Clontech include KC8 competent cells, anti-LexA monoclonal antibodies, a yeast transformation system, a yeast plasmid isolation kit, and an EGY48 partner strain for yeast mating to facilitate the analysis of interaction specificity. OriGene has a generally similar product line to Clontech. In contrast, Invitrogen has substantially modified the Interaction Trap core reagents to develop its own bait and library plasmids. pHybLex/Zeo, a novel bait plasmid, is ~50% smaller than the original pEG202 (making it easier to clone into), and it has an enriched polylinker. Significantly, it replaces both the Ap^r and *HIS3* genes with a novel gene that confers resistance to the antibiotic Zeocin (supplied with the kit), which provides selection in both bacteria and yeast. This elimination of auxotrophic selection for the bait plasmid renders the LexA-fusion construct usable with libraries and strains from all existing two-hybrid systems and additionally facilitates the direct selection of library plasmid in strains other than KC8. Some changes, which are designed to make the vector easier to use, have also been introduced in the library vector pYESTrp (e.g., it uses a V5 epitope tag for protein detection). The Invitrogen kit, termed Hybrid Hunter, includes the bait/library/reporter plasmids and EGY48 yeast strain as noted, and additionally includes primer sets for bait and library plasmids and the L40 yeast strain, should an investigator wish to use a *HIS3* auxotrophy selection. Additional related products from Invitrogen include antibodies for detection of bait and prey fusion proteins (anti-LexA and anti-V5), pJG4-5 library vector primers, and a Transformation Kit.

A significant advantage of the entry of commercial entities into the Interaction Trap field is the rapid increase in the number of compatible cDNA libraries. A list of currently available premade libraries available from these companies is presented in Table 20.1.3, and custom-made libraries are also available upon request. Because new libraries and other related reagents are being constantly added to the line of two-hybrid related products, it is advisable to contact the companies or visit their Web sites (*www.clontech.com, www.invitrogen.com,* and *www.origene.com*) for the latest information.

Finally, over the last several years, a number of groups have adapted basic two-hybrid strategies to more specialized applications, and they have devised strategies to broaden their basic functionality. Interaction Mating (Finley and Brent, 1994) has been used to establish extended networks of targeted protein-protein interaction. In this approach, a panel of LexA-fused proteins are transformed into a *MATa* haploid selective strain (such as RFY206), a panel of activation-domain fused proteins are transformed into a suitable MATα haploid (such as EG448), and the two panels are cross-gridded against each other for mating. Selected diploids are then screened by replica plating to selective medium. This approach complements library screening in large-scale applications, such as proposed definition of interaction maps for entire genomes (Bartel et al., 1996).

Interaction mating has also provided the basis for an alternative two-hybrid hunt protocol (see Alternate Protocol 2), useful in cases when a single library will be screened with different baits. In this approach (Bendixen et al., 1994; Finley and Brent, 1994: Kolonin and Finley, 1998), a library is introduced into a single strain, like EGY48, and aliquots are stored frozen. To conduct a hunt, an aliquot is thawed and mated with a strain expressing a bait. This allows one to avoid repeated high-efficiency transformations, since a single library transformation can provide enough pretransformed yeast to conduct dozens of interactor hunts. Moreover, some yeast strains pretransformed with libraries are becoming commercially available, which may eliminate altogether the need to conduct a high-efficiency library transformation for some researchers.

Two-hybrid approaches have been shown to be effective in identifying small peptides with biological activities on selected baits (Yang et al., 1995; Colas et al., 1996), which may prove to be useful as a guide to targeted drug design. Rapid screening protocols have been devised using custom-synthesized libraries expressing sheared plasmid DNA to facilitate rapid mapping of interaction interfaces (Stagljar et al., 1996). Osborne and coworkers have demonstrated the effectiveness of a tribrid (or tri-hybrid) approach, in which an additional plasmid expresses a tyrosine kinase to specifically modify a bait protein, allowing detection of SH2-domain-containing partner proteins that recognize specific phosphotyrosine residues (Osborne et al., 1995). A variety of more elaborate tribrid approaches, in which a DNA-binding domain fused protein is used to present an intermediate nonprotein compound for interaction with a library, have been developed and proven effective. These approaches have allowed the identification of proteins binding specific drug ligands (Chiu et al., 1994; Licitra and Liu, 1996), as well as the identification of proteins binding to RNA sequences (SenGupta et al., 1996; Wang et al., 1996). It is expected that the range of utility of these systems will continue to expand.

Critical Parameters and Troubleshooting

To maximize chances of a successful interactor hunt, a number of parameters should be taken into account. Before attempting a screen, bait proteins should be carefully tested to ensure that they have little or no intrinsic ability to activate transcription. Bait proteins must be expressed at reasonably high levels and must be able to enter the yeast nucleus and bind DNA (as confirmed by the repression assay). Optimally, integrity and levels of bait proteins should be confirmed by immunoblot analysis, using an antibody to either LexA or the fused domain. In particular, at this time, bait proteins that have extensive transmembrane domains or are normally excluded from the nucleus are not likely to be productively used in a library screen. Proteins that are moderate to strong activators will need to be truncated to remove activating domains before they can be used.

If a protein neither activates nor represses, the most likely reason is that it is not being made. This can be determined by immunoblot analysis of a crude lysate protein extract of EGY48 (*UNIT 10.8*; Samson et al., 1989) containing the plasmid, using anti-LexA antibodies as primary antiserum. If the full protein is not made, it may be possible to express truncated derivatives of the protein. If the protein is made, but still does not repress, it may not enter the yeast nucleus effectively, although this appears to be a relatively rare problem. In this case, introducing the coding sequence for the fused moieity into a LexA fusion vector containing a nuclear localization motif (e.g., pJK202; J. Kamens, BASF, Worcester, Mass., unpub. observ.) may solve the problem.

The test for the leucine (Leu) requirement is extremely important to determine whether the bait protein is likely to yield an unworkably high background. The *LEU2* reporter in EGY48 is more sensitive than the pSH18-34 reporter for some baits (Estojak et al., 1995). Therefore, it is possible that a bait protein demonstrating little or no signal in a β-galac-

tosidase assay may nevertheless permit some growth on –Leu medium. If this occurs, there are several options. First, a less sensitive strain can be used, as described in the text. Second, background can sometimes be reduced further by making the EGY strain diploid (e.g., D. Krainc, Harvard Medical School, Boston, Mass.; R. Finley and R. Brent, unpub. observ.) or by performing the hunt by interaction mating as described in Alternate Protocol 2. A third option is to attempt to truncate the bait protein to remove activating function. In general, it is useful to extrapolate from the number of cells that grow on –Leu medium to the number that would be obtained in an actual library screen, and determine if this is a background level that can be tolerated. For example, if two colonies arise from 100,000 plated cells on –Leu medium, 200 to 400 would be expected in an actual screen of 10^6 cDNAs. Although this is a high initial number of positives, the vast majority should be eliminated immediately through easily performed controls. This is a judgment call. Finally, very rarely it happens that a bait that appears to be well behaved and negative for transcriptional activation through all characterization steps will suddenly develop a very high background of transcriptional activation following library transformation. The reason for this is currently obscure, and no means of addressing this problem has as yet been found: such baits are hence inappropriate for use in screens.

The protocols described in this unit use initial screening with the most sensitive reporters followed by substitution with less sensitive reporters if activation is detected. An obvious question is, why not start out working with extremely stringent reporters and know immediately whether the system is workable? In fact, some researchers routinely use a combination of pJK103 or pRB1840 with EGY191, and obtain proteins that to date appear to be biologically relevant partners from library screens. However, extensive comparison studies using interactors of defined in vitro affinity with different combinations of *LacZ* and *LEU2* reporters (Estojak et al., 1995) have indicated that although the most sensitive reporters (pSH18-34) may in some cases be prone to background problems, the most stringent reporters (EGY191, pRB1840) may miss some interactions that certainly are biologically relevant and occur inside cells. In the end, the choice of reporters devolves to the preference of individual investigators: the bias of the authors is to cast a broad net in the early stages of a screen, and hence to use more sensitive reporters when practicable.

It is important to move expeditiously through characterization steps and to handle yeast transformed with bait plasmids with care. In cases where yeasts have been maintained on plates for extended periods (e.g., 4 days at room temperature or >2 to 3 weeks at 4°C), unexpected problems may crop up in subsequent library screens.

The transformation protocol is a version of the lithium acetate transformation protocol described by Schiestl and Gietz (1989) and Gietz et al. (1992; see *UNIT 13.7*) that maximizes transformation efficiency in *Saccharomyces cerevisiae* and produces up to 10^5 colonies/μg plasmid DNA. In contrast to *Escherichia coli*, the maximum efficiency of transformation for *S. cerevisiae* is ~10^4 to 10^5/μg input DNA. It is extremely important to optimize transformation conditions before attempting an interactor hunt. Perform small-scale pilot transformations to ensure this efficiency is attained and to avoid having to use prohibitive quantities of library DNA. In addition, as for any effort of this type, it is a good idea to obtain or construct a library from a tissue source in which the bait protein is known to be biologically relevant.

In practice, the majority of proteins isolated by interaction with a LexA fusion turn out to be specific for the fused domain; a smaller number are nonspecifically sticky, and to date there appears to have been only one isolation from a eukaryotic library of a protein specific for LexA. However, it is generally informative to retest positive clones on more than one LexA bait protein; ideally, library-derived clones should be tested against the LexA fusion used for their isolation, several LexA fusions to proteins that are clearly unrelated to the original fusion, and if possible, several LexA fusions that there is reason to believe are related to the initial protein (e.g., if the initial probe was LexA-Fos, a good related set would include LexA-Jun and LexA-GCN4).

Colony selection for master plate production is one of the more variable parts of the procedure. For strong interactors, colonies will grow up in 2 days. However, if plates are left at 30°C, new colonies will continue to appear every day. Those that appear rapidly are most likely to reflect interactors that are biologically relevant to the bait protein. Those that appear later may or may not be relevant. However, many parameters can delay the time of colony formation of cells that contain valid interactions, including the strength of the interaction

and the level of expression of the library-encoded protein.

Anticipated Results

Depending on the protein used as bait, anywhere from zero to hundreds of specific interactors will be obtained from 10^6 primary transformants.

Time Considerations

If all goes well, once the required constructions have been made it will take ~1 week to perform yeast transformations, obtain colonies, and determine whether bait proteins are appropriate. It will take a second week to perform library transformations, replate to selective medium, and obtain putative positives. A third week will be required to rescue the plasmid from the yeast, passage it through *E. coli*, transform fresh yeast, and confirm specificity.

Literature Cited

Bartel, P.L., Chien, C.-T., Sternglanz, R., and Fields, S. 1993. Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach (D.A. Hartley, ed.) pp. 153-179. Oxford University Press, Oxford.

Bartel, P.L., Roecklein, J.A., SenGupta, D., and Fields, S. 1996. A protein linkage map of *Escherichia coli* bacteriophage T7. *Nature Genet.* 12:72-77.

Bendixen, C., Gangloff, S., and Rothstein, R. 1994. A yeast mating-selection scheme for detection of protein-protein interactions. *Nucl. Acids Res.* 22:1778-1779.

Breeden, L. and Nasmyth, K. 1985. Regulation of the yeast HO gene. *Cold Spring Harbor Symp. Quant. Biol.* 50:643-650.

Brent, R. and Ptashne, M. 1984. A bacterial repressor protein or a yeast transcriptional terminator can block upstream activation of a yeast gene. *Nature* 312:612-615.

Brent, R. and Ptashne, M. 1985. A eukaryotic transcriptional activator bearing the DNA specificity of a prokaryotic repressor. *Cell* 43:729-736.

Chien, C.-T., Bartel, P.L., Sternglanz, R., and Fields, S. 1991. The two-hybrid system: A method to identify and clone genes for proteins that interact with a protein of interest. *Proc. Natl. Acad. Sci. U.S.A.* 88:9578-9582.

Chiu, M.I., Katz, H., and Berlin, V. 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. *Proc. Nat. Acad. Sci. U.S.A.* 91:12574-12578.

Colas, P., Cohen, B., Jessen, T., Grishina, I., McCoy, J., and Brent, R. 1996. Genetic selection of peptide aptamers that recognize and inhibit cyclin-dependent kinase 2. *Nature* 380:548-550.

Durfee, T., Becherer, K., Chen, P.L., Yeh, S.H., Yang, Y., Kilburn, A.E., Lee, W.H., and Elledge, S.J. 1993. The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. *Genes & Dev.* 7:555-569.

Estojak, J., Brent, R., and Golemis, E.A. 1995. Correlation of two-hybrid affinity data with in vitro measurements. *Mol. Cell. Biol.* 15:5820-5829.

Fearon, E.R., Finkel, T., Gillison, M.L., Kennedy, S.P., Casella, J.F., Tomaselli, G.F., Morrow, J.S., and Dang, C.V. 1992. Karyoplasmic interaction selection strategy: A general strategy to detect protein-protein interactions in mammalian cells. *Proc. Nat. Acad. Sci. U.S.A.* 89:7958-7962.

Fields, S. and Song, O. 1989. A novel genetic system to detect protein-protein interaction. *Nature* 340:245-246.

Finley, R.L., Jr., and Brent, R. 1994. Interaction mating reveals binary and ternary connections between *Drosophila* cell cycle regulators. *Proc. Natl. Acad. Sci. U.S.A.* 91:12980-12984.

Gietz, D., St. Jean, A., Woods, R.A., and Schiestl, R.H. 1992. Improved method for high-efficiency transformation of intact yeast cells. *Nucl. Acids Res.* 20:1425.

Golemis, E.A. and Brent, R. 1992. Fused protein domains inhibit DNA binding by LexA. *Mol. Cell Biol.* 12:3006-3014.

Grunstein, M., and Hogness, D.S. 1975. Colony hybridization: A method for the isolation of cloned DNAs that contain a specific gene. *Proc. Natl. Acad. Sci. U.S.A.* 72:3961-3965.

Gyuris, J., Golemis, E.A., Chertkov, H., and Brent, R. 1993. Cdi1, a human G1- and S-phase protein phosphatase that associates with Cdk2. *Cell* 75:791-803.

Kaiser, C., Michaelis, S., and Mitchell, A. 1994. Methods in Yeast Genetics, a Cold Spring Harbor Laboratory Course Manual, pp.135-136. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

Kolonin, M.G. and Finley, R.L., Jr. 1998. Targeting cyclin-dependent kinases in *Drosophilia* with peptide aptamers. *Proc. Natl. Acad. Sci. U.S.A. In press.*

Licitra, E.J. and Liu, J.O. 1996. A three-hybrid system for detecting small ligand-protein receptor interactions. *Proc. Nat. Acad. Sci. U.S.A.* 93:12817-12821.

Ma, J. and Ptashne, M. 1987. A new class of yeast transcriptional activators. *Cell* 51:113-119.

Ma, J. and Ptashne, M. 1988. Converting an eukaryotic transcriptional inhibitor into an activator. *Cell* 55:443-446.

Osborne, M., Dalton, S., and Kochan, J.P. 1995. The yeast tribrid system: Genetic detection of trans-phosphorylated ITAM-SH2 interactions. *Bio/Technology* 13:1474-1478.

Ruden, D.M., Ma, J., Li, Y., Wood, K., and Ptashne, M. 1991. Generating yeast transcriptional activators containing no yeast protein sequences. *Nature* 350:426-430.

Samson, M.-L., Jackson-Grusby, L., and Brent, R. 1989. Gene activation and DNA binding by

Drosophila Ubx and abd-A proteins. *Cell* 57:1045-1052.

Schiestl, R.H. and Gietz, R.D. 1989. High-efficiency transformation of intact yeast cells using single-stranded nucleic acids as a carrier. *Curr. Genet.* 16:339-346.

SenGupta, D.J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. 1996. A three-hybrid system to detect RNA-protein interactions in vivo. *Proc. Nat. Acad. Sci. U.S.A.* 93:8496-8501.

Sikorski, R.S. and Hieter, P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae. *Genetics* 122:19-27.

Stagljar, I., Bourquin, J.-P., and Schaffner, W. 1996. Use of the two-hybrid system and random sonicated DNA to identify the interaction domain of a protein. *BioTechniques* 21:430-432.

Vasavada, H.A., Ganguly, S., Germino, F.J., Wang, Z.X., and Weissman, S.M. 1991. A contingent replication assay for the detection of protein-protein interactions in animal cells. *Proc. Nat. Acad. Sci. U.S.A.* 88:10686-10690.

Vojtek, A.B., Hollenberg, S.M., and Cooper, J.A. 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. *Cell* 74:205-214.

Wang, Z.F., Whitfield, M.L., Ingledue, T.C.3, Dominski, A., and Marzluff, W.F. 1996. The protein that binds the 3′ end of histone mRNA: A novel RNA-binding protein required for histone pre-mRNA processing. *Genes & Dev.* 10:3028-3040.

Watson, M.A., Buckholz, R., and Weiner, M.P. 1996. Vectors encoding alternative antibiotic resistance for use in the yeast two-hybrid system. *BioTechniques* 21:255-259.

West, R.W.J., Yocum, R.R., and Ptashne, M. 1984. *Saccharomyces cerevisiae GAL1-GAL10* divergent promoter region: Location and function of the upstream activator sequence UASG. *Mol. Cell Biol.* 4:2467-2478.

Yang, M., Wu, Z., and Fields, S. 1995. Protein-peptide interactions analyzed with the yeast two-hybrid system. *Nucl. Acids Res.* 23:1152-1156.

Key Reference

Gyuris et al., 1993. See above.

Initial description of interaction trap system.

Internet Resources

http://www.clontech.com

http://cmmg.biosci.wayne.edu/rfinley/lab.html

Source of two-hybrid information, protocols, and links.

http://www.invitrogen.com

http://www.origene.com

Commercial sources for basic plasmids, strains, and libraries for interaction trap experiments.

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Sources of interaction trap plasmids for specialized interactions.

http://www.fccc.edu:80/research/labs/golemis/InteractionTrapInWork/html

Database for false positive proteins detected in interaction trap experiments; analysis of two-hybrid usage.

http://xanadu.mgh.harvard.edu/brentlabhome page4.html

Database of interaction trap protocols and related issues.

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